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ABSORPTION OF SUBCUTANEOUSLY IMPLANTED HORMONE PELLETS

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IN 1937, Deanesly and Parkes showed experimentally on animals that steroid hormones can be administered in the form of subcutaneously implanted tablets or pellets, and that the resultant physiologic action is continuous, intense, and prolonged. The availability of numerous hormones and their esters with wide ranges of absorbability and potency permitted them to predict that "additional information should thus make possible treatment of almost any intensity and duration by a single administration" of steroid hormones in this form (Deanesly and Parkes, 1938).

The technic was rapidly and widely applied to experimental work with hormones in animals, and many interesting results and applications have been recorded. The work of Biskind (Biskind and Meyer, 1941; Mark and Biskind, 1941; Biskind *et al.*, 1941), Emmens (1941) and Forbes (1941–42) particularly clarified the factors involved in the dose and effect of hormones administered in the form of solid pellets. The rate of absorption of estrogen and androgen pellets in rats was not affected by the site of subcutaneous implantation, physiologic need, sex, or the simultaneous introduction of both types of hormones (Mark and Biskind, 1941). The qualitative physiologic effect of hormones was the same as when they were administered in oil solutions, but the potency of pellets of various esters of the hormones could not be evaluated by the comparative potency of the chemicals given in oily media (Biskind and Meyer, 1941). The administration of hormones in pellet form was uniformly more effective per unit weight than the usual parenteral route, due to the continuous absorption. In rats, the absorption rate from uniform compressed pellets was repro-

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ducible within ten per cent of the mean for most of the estrogens and androgens (Emmens, 1941). Pellets made by compression of large crystals absorbed at the same rate as pellets made of small crystals (Forbes, 1942), and no difference in absorption rate was found between pellets made by compression or by fusion (Deanesly and Parkes, 1943). One of the main factors controlling the rate of absorption was found to be the surface area exposed to the tissue fluids (Biskind *et al.*, 1941; Forbes, 1941). Although the thickness of the fibrous capsule forming around the pellet, as well as the protein material that penetrates into the pellet, have been stated to inhibit the rate of absorption, the experimental work of Emmens (1941) and Deanesly and Parkes (1943) does not support the contention.

The administration of steroid hormones in the form of subcutaneously implanted pellets was used clinically by Bishop in 1938, and since then a considerable literature has accumulated. Hormone pellets have been used in the treatment of adrenal cortical insufficiency (Thorn *et al.*, 1939, 1942), in spontaneous and induced menopause (Bennett *et al.*, 1940; Twombly and Millen, 1941), in eunuchism and eunuchoidism (Escamilla and Lisser, 1941; Howard and Jewett, 1942), and in prostatic carcinoma (Kearns, 1942; Shimkin and Zon, 1943). The work of Thorn and his associates has established that the subcutaneous implantation of desoxycorticosterone pellets, combined with other measures, is an excellent form of treatment in Addison's disease. With estrogens and with androgens, however, the early encouraging results have been followed by less favorable reports.

Examination of reports on the clinical use of estrogens and androgen pellets indicates that difficulties have been encountered particularly under two conditions: (1) with the use of very slowly absorbable materials, such as estradiol benzoate, and (2) with the use of large pellets, weighing over 200 mg. It was found that in these cases the therapeutic effect was relatively short-lived although sizable pellets were still retained. Many clinical writers have attributed this effect to the formation of a fibrous capsule around the pellets, with presumed resultant inhibition of absorption (Geist *et al.*, 1940; Escamilla and Lisser, 1941; Howard and Jewett, 1942). This interpretation is not substantiated experimentally, and it is just as likely that the thickness of the capsule is determined by the slow absorbability of the material rather than the slowness of absorption being due to the deposition of the surrounding fibrous tissue. Moreover, it is evident that in the interpretation of the findings, the physical laws governing the absorption of the implanted pellets, particularly the surface area-volume relationships, have not been adequately considered.

The experiments reported here demonstrate that the decreased rate of absorption of subcutaneously implanted pellets is attributable simply to the manner in which the surface area of such pellets changes with changes in volume. It is substantiated that, with the factors of

density, solubility, and the recipient being constant, the governing factor controlling the rate of absorption is the surface area of the pellet, which depends on the shape and the volume of the pellet.

EXPERIMENTAL PROCEDURE

Diethylstilbestrol,[2] melting point 169–171 degrees C. (corrected), was melted over an oil bath, and drawn up into thick-walled glass tubes equipped with well-fitting metal plungers. Upon solidification, the material was extruded and the resultant solid strings were cut with a razor blade into segments of desired length (Shimkin and White, 1941). No lubricant was used in the glass tubes, and after cutting, the pellets were washed lightly with absolute alcohol. The walls of the cylinders were very smooth, but the cut ends were somewhat irregular in comparison. Pellets measuring 1.1×1.1 mm. 2.4×2.4 mm., and 3.1×3.1 mm. and weighing approximately 1.2, 12.5, and 27.5 mg., respectively, were thus prepared.

The preparation of hormone pellets by fusion rather than by compression has several advantages, the chief one for clinical use being the preparation under sterilizing conditions. By maintaining the heat of melted diethylstilbestrol at 175–180 degrees C. for 20 minutes after the addition of 10 million spores of B. tetanus per 100 mg. of the chemical, the pellets were noninfective to guinea pigs into which they were implanted.[3] In these experiments, fused pellets were advantageous because their surfaces tend to become less eroded than the usual compressed pellets, and little or no protein material deposition occurs within the pellets (Deanesly and Parkes, 1943).

The pellets were introduced through trocars of appropriate size into the subcutaneous tissue of the right axilla of adult male mice of strain *dba*, weighing approximately 30 grams. At varying intervals, the pellets were removed, carefully freed of connective tissue, dried thoroughly, and weighed. The pellets were then reimplanted into other mice of the same strain and sex. Previous data[4] with pellets weighing 1.0 and 7.5 mg. showed that the rate of absorption was the same whether the pellets were removed at intervals or were permitted to remain *in situ*. The formation of a connective tissue capsule, which was always well vascularized, had no effect upon the absorption of diethylstilbestrol pellets.

RESULTS

The absorption rate of 21 pellets, determined from 108 observations, was established. The data are presented in table 1, and plotted in figs. 1, 2, and 3. The solid lines in these figures represent the theoretical absorption curves, calculated from the experimental data according to the following computations.

It was assumed that the rate of absorption of a pellet is a function of the area of the pellet exposed to the solvent action of the body fluids. If this solvent action remains constant during the time of

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[2] We are indebted to Merck and Co. for a generous supply of the chemical.

[3] Shimkin, M. B., unpublished data, 1942.

[4] Shimkin, M. B., and Zon, L., unpublished data 1942.

TABLE 1. ABSORPTION OF SUBCUTANEOUSLY IMPLANTED PELLETS OF DIETHYLSTILBESTROL

Pellet No.	Original size, d×l mm.	Original weight mg.	Weight of pellet, in mg. / Time implanted, in days.									
1	1.1×1.1	1.3	1.1 / 3	0.6 / 13								
2	1.1×1.1	1.1	1.0 / 3	0.5 / 13								
3	1.1×1.1	1.4	1.1 / 5	0.5 / 15	0.4 / 22	0.3 / 28	0.2 / 37					
4	1.1×1.1	1.1	0.9 / 5	0.5 / 15	0.3 / 30							
5	1.1×1.1	1.2	0.3 / 29	0.2 / 34	0.1 / 37							
6	1.1×1.1	1.0	0.7 / 8	0.3 / 32								
7	1.1×1.1	1.0	0.6 / 10	0.5 / 17	0.2 / 31							
8	2.4×2.4	12.6	11.6 / 3	9.5 / 16	8.4 / 25	7.3 / 35	3.6 / 67	3.3 / 85	2.2 / 97	1.6 / 102	1.0 / 113	0.4 / 123
9	2.4×2.4	13.0	12.0 / 3	9.8 / 13	7.3 / 37	5.1 / 56	4.1 / 71	3.0 / 102	2.5 / 117	1.7 / 129		
10	2.4×2.4	12.4	11.6 / 5	8.8 / 25	6.3 / 48	4.5 / 72	2.5 / 95	1.8 / 109	1.3 / 117	0.8 / 126		
11	2.4×2.4	12.6	11.6 / 5	6.7 / 32	4.7 / 48	2.6 / 69	1.5 / 86	1.1 / 93	1.0 / 98	0.7 / 105	0.2 / 108	
12	2.4×2.4	12.8	11.2 / 7	9.3 / 20	8.3 / 32	6.2 / 51	4.8 / 69	4.2 / 79	3.1 / 102	2.2 / 123		
13	2.4×2.4	12.1	6.0 / 31	4.7 / 42	3.9 / 51							
14	2.4×2.4	12.2	4.0 / 36	3.0 / 61	1.5 / 65	1.3 / 71	1.2 / 75	0.6 / 86	0.3 / 96			
15	3.1×3.1	27.4	26.5 / 3	22.4 / 13	20.8 / 22	14.8 / 45	11.7 / 61	7.8 / 83	6.8 / 92	4.4 / 114		
16	3.1×3.1	27.6	26.6 / 3	22.0 / 13	18.2 / 29	15.0 / 51	13.0 / 73	12.3 / 80				
17	3.1×3.1	29.8	28.3 / 5	24.0 / 15	19.0 / 38							
18	3.1×3.1	28.0	25.7 / 7	21.9 / 20	17.5 / 37	16.3 / 42	13.4 / 57	9.0 / 80	6.0 / 102	5.0 / 109		
19	3.1×3.1	29.0	25.3 / 7	21.4 / 23	19.9 / 29	18.0 / 35	15.5 / 53	14.8 / 60				
20	3.1×3.1	25.0	15.6 / 35	13.6 / 45	9.8 / 69							
21	3.1×3.1	27.0	25.0 / 5									

implantation, and if the pellet retains approximately its original form, the absorption rate is expressed by the equation:

$$\frac{dV}{dt} = -KA$$

where V is the volume, A the area, t the time, and K the constant of absorption. In the case of a spherical pellet of radius r,

$$\frac{d(4/3\pi r^3)}{dt} = -K4\pi r^2$$

$$dr = -K\,dt$$

$$r = r_0 - Kt \qquad (1)$$

where r_0 is the radius at $t = 0$ and K is a constant equal to the decrease

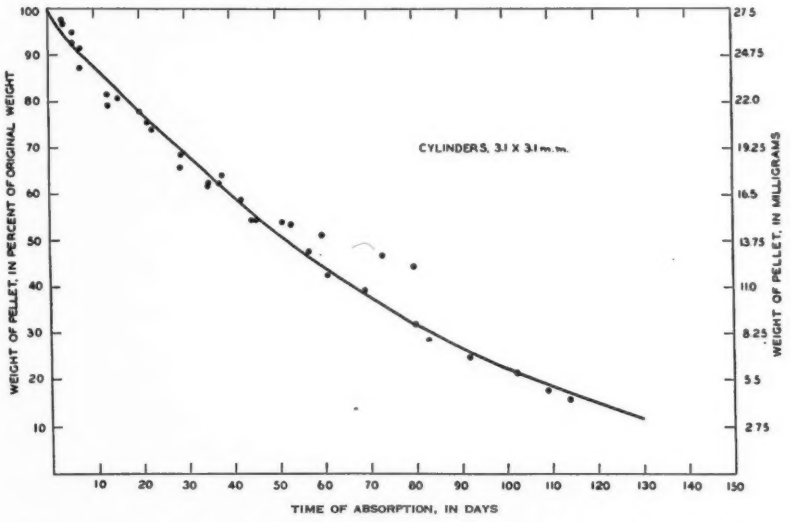

FIG. 1. Absorption of diethylstilbestrol pellets in mice. Average weight of pellets, 27.5 mg.; dimensions, 3.1×3.1 mm.

in the radius per unit time (day). Then the values for volume, V, or weight, W, respectively, for any time, t, take the form:

$$V = 4/3\pi(r_0 - Kt)^3$$

$$W = 4/3\pi\rho(r_0 - Kt)^3 \qquad (2)$$

where ρ is the density.

The above principles also hold for cylindrical pellets, if the slight rounding off effect of the body fluids upon the edges of such pellets is neglected.

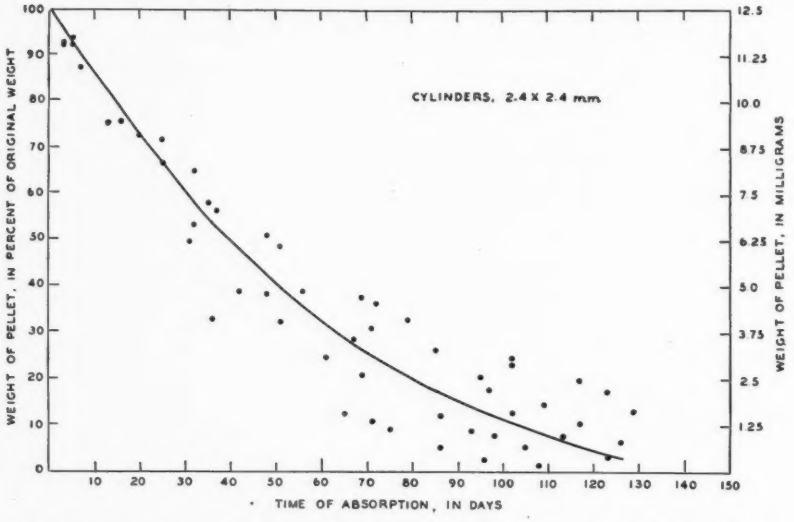
CYLINDERS, 2.4 X 2.4 mm.
WEIGHT OF PELLET, IN PERCENT OF ORIGINAL WEIGHT
WEIGHT OF PELLET, IN MILLIGRAMS
TIME OF ABSORPTION, IN DAYS

FIG. 2. Absorption of diethylstilbestrol pellets in mice. Average weight of pellets, 12.5 mg.; dimensions, 2.4×2.4 mm.

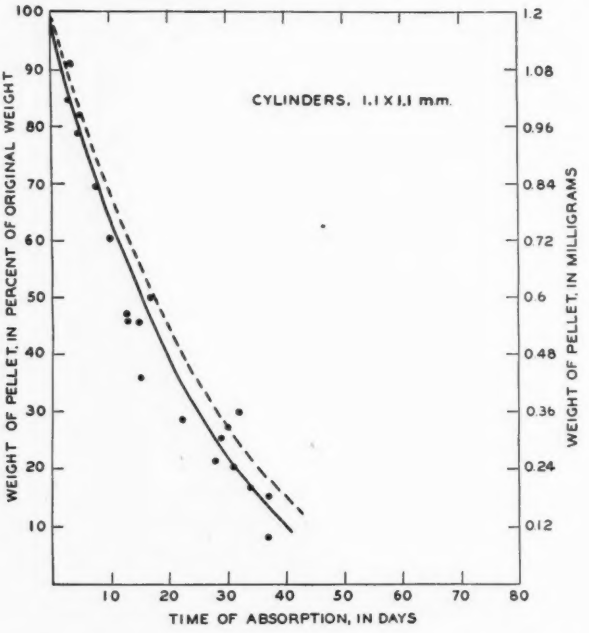
CYLINDERS, 1.1 X 1.1 mm.
WEIGHT OF PELLET, IN PERCENT OF ORIGINAL WEIGHT
WEIGHT OF PELLET, IN MILLIGRAMS
TIME OF ABSORPTION, IN DAYS

FIG. 3. Absorption of diethylstilbestrol pellets in mice. Average weight of pellets, 1.2 mg.; dimensions, 1.1×1.1 mm. The solid line best fits the data, but the broken line which best fits the combined data of figures 1, 2, and 3 was used.

$$W = \pi\rho hr^2$$

$$h = br$$

where h is the height of the cylinder and b a constant expressing the relationship between h and r. Replacing r by (1),

$$W = \pi\rho(r_0 - Kt)^2\,(br_0 - 2Kt) \tag{3}$$

Three special cases in relation to the shape of the cylinder are solved as follows:

I. $b = 2$; the height of the cylinder equals its diameter.

$$W = 2\pi\rho(r_0 - Kt)^3 \tag{4}$$

$$\sqrt[3]{W} = \sqrt[3]{W_0} - ct. \tag{4a}$$

$$c = \sqrt[3]{2\pi\rho}\cdot K$$

Therefore, the cube root of the weight of the pellet plotted against time is a straight line. This also holds for a spherical pellet with $c = \sqrt[3]{\frac{4}{3}\pi\rho}K$, the constant c being the cube root of a weight per unit time.

II. $b \gg 1$; the height of the cylinder is large in comparison with the radius. In this case, the length of the cylinder will change only slightly with time and equation (3) can be written approximately as

$$W = \pi\rho br_0(r_0 - Kt)^2 \tag{5}$$

III. $b \ll 1$; the height of the cylinder is small in comparison with the radius:

$$W = \pi\rho r_0^2(br_0 - Kt) \tag{6}$$

Since fused cylindrical pellets are easy to prepare, and since the calculations involved in cylindrical pellets in which the height of the pellet is equal to the diameter are the simplest, this type of pellet was employed in these experiments.

The theoretical absorption curves of figs. 1, 2, and 3 can be obtained as follows: A value for c is obtained from equation (4a).

$$\frac{\sqrt[3]{W_0} - \sqrt[3]{W}}{t} = c \tag{7}$$

where W_0 is the original weight of the pellet, W the weight at time t (in days) as determined from the experimental data. With this value of c and with slightly different values of c a series of curves is calculated and the curve best fitting the experimental selected. The original weights of the three sizes of pellets used were 1.2, 12.5 and 27.5 mg., and the data were plotted with different ordinates for better comparison. All these data should lie on a common curve. The curve in fig. 4 gives the combined curve, which reproduces the curve of fig. 1 with the curves for smaller pellets beginning at the point where the

larger pellet has been absorbed to this size. The dotted curve of fig. 3, lying slightly above the actual curve that best fits the data, was used; the small size of the original pellets made errors in weighing inevitable.

The values of the constants of equations (4) and (4a) as determined from the combined experimental data are:

$$K = 6 \times 10^{-4} \text{ cm/day}$$

$$c = 1.2 \times 10^{-2} \text{ gm}^{1/3}\text{/day},$$

with the density $\rho = 1.18$ gm/cm^3. The constant K gives the decrease of the radius in cm. per day.

For practical purposes, it is not necessary to go through the pro-

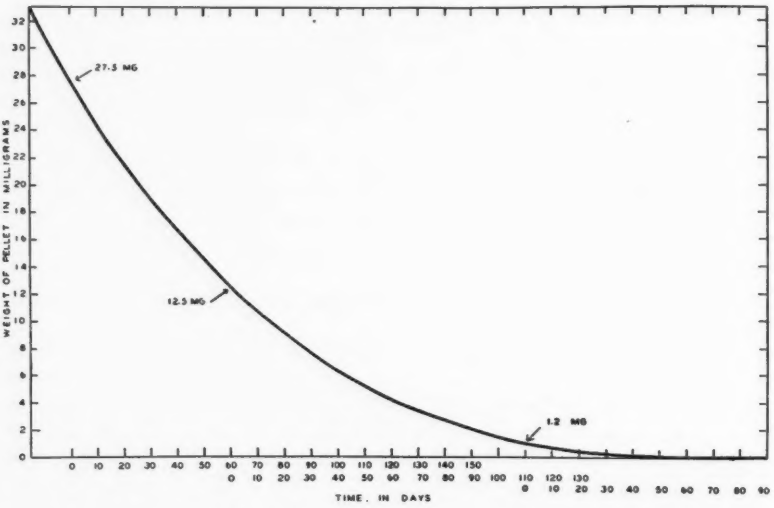

FIG. 4. Absorption of diethylstilbestrol pellets in mice, where cylindrical pellets with $h = 2r$ are used.

cedure just outlined. It is sufficient to plot the cube root of the weight of the pellets, as determined by experimental observations, against time and to draw a straight line through the data. As $d\sqrt[3]{W}/dt = -c$, the constant c is the slope of the line (equation 7). Pellets of different weight will give parallel lines, even if there is a variation in the density of the pellets, as $\sqrt[3]{\rho K}$ remains constant. This holds true only for spherical and cylindrical pellets in which $h = 2r$.

DISCUSSION

The foregoing results and calculations show that, in mice, the absorption of subcutaneously implanted pellets is a function of the surface area. With a gradual reduction of the size of the pellet, the

rate of absorption decreases because the surface area is reduced. Disc-shaped pellets, as pointed out by Emmens (1941), have the greatest tendency to a constant rate of absorption. It is evident that in order to maintain an approximately constant level of dosage of the hormone for a protracted period of time, it is necessary to implant additional pellets to compensate for the absorption, no matter what shape of pellet is used. The physical laws governing the absorption explain the decreased rate of absorption without recourse to additional factors such as the formation of a fibrous capsule around the pellet.

In view of the above information, two sets of clinical data on the absorption of androgen and estrogen pellets were examined.

Diethylstilbestrol. Shimkin and Zon (1943) published a curve for the absorption of diethylstilbestrol pellets in man, in which cylindrical pellets of 50 mg., 2.7 mm. in diameter and 8.5 mm. in height, were employed. As previously stated, the simple equations for $h_0 = 2r_0$ pellets do not apply for the absorption of pellets of the shape employed. However, an approximate value for the constant $c = 1.2 \times 10^{-2} \text{gm.}^{1/3}$ per day, sufficiently accurate for practical purposes, was calculated. The constant is the same for human beings as for mice.

With this numerical value for the constant, the absorption per day of cylindrical pellets with $h = 2r$ can be calculated from equation (4a). It was found that such a pellet, weighing 25 mg., will give an initial absorption rate of 0.31 mg. Four pellets, therefore, will yield an initial absorption of 1.24 mg. per day. At the end of 50 days, the absorption rate will drop to 0.8 mg. per day. The supplement of another pellet at this time will increase the dosage to 1.1 mg., which 30 days later again will drop to 0.8 mg. per day. The additional implantation of one 25 mg. pellet, the first after 50 days, and then every 30 days, therefore, will maintain an absorption rate fluctuating between 1.1 and 0.8 mg. per day for an indefinite period of time. Figure 5 illustrates the absorption rate with the outlined procedure.

There are, of course, variations in the absorption rate in different individuals. Theoretically, if the absorption rate in an individual is lower than calculated, the addition of a new pellet at 50 days will not raise the dosage above the initial value because the absorption rate of the new pellet will also be lower. The mean absorption rate will remain near the calculated value but the fluctuations will be smaller. With an initial absorption rate higher than calculated, the mean rate will also remain near the calculated value but the fluctuations will be greater. This holds only if the absorption rate varies within a fairly narrow range (e.g., plus-minus 10 per cent) and if this rate remains fairly constant in the individual.

It is difficult to achieve a constant absorption rate with single implantation of pellets, but this can be approached by the periodic reimplantation of additional pellets. The decreased rate of absorption, however, may be useful in the administration of estrogens in this form

to women, since it allows the reproduction of the normal cyclic variations in estrogenic stimulation. It has been established that continual estrogen treatment of menopausal women, at least in those in whom the uterus has not been removed, is undesirable because it leads to endometrial hyperplasia and eventual bleeding (Twombly and Millen, 1941). Cyclic therapy can be achieved by the use of sufficiently small pellets of an estrogen with a fairly rapid rate of absorption, such as diethylstilbestrol. For example, the implantation of 25 pellets of diethylstilbestrol weighing 0.5 mg. and being 0.5×0.5 mm. in size, will yield a mean dose of 0.5 mg. per day for 10 days and gradually decrease to 0.1 mg. on the 38th day. After an appropriate rest period, the

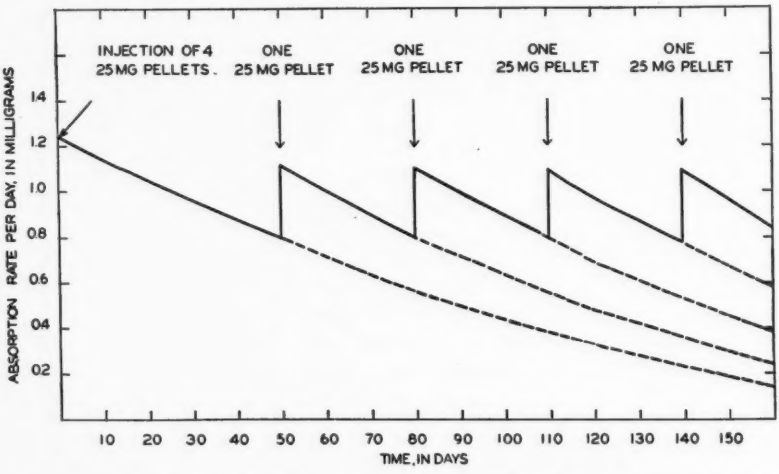

FIG. 5. Repeated implantations of diethylstilbestrol pellets as a means of establishing a continuous absorption rate of 1 mg. per day, in man. Computed from data of Shimkin and Zon.

procedure can be repeated. The small size of the pellets would make it possible to introduce them through a hypodermic needle of reasonable gage and would require no operative procedure.

Testosterone propionate. Howard and Jewett (1942) have published the absorption curve of testosterone propionate pellets. The mean weight of the pellets was 202.6 mg. They were cylinders with rounded ends, measuring 6.5 mm. in diameter and 6.5 mm. in height. As these pellets obey the $h=2r$ relation, the data will be represented by a straight line when the cube root of the weight is plotted against time (Fig. 6). The constant c for the mean of the data is found to be 0.9×10^{-2} $gm^{1/3}$ per day. It is clear that the decrease in the absorption rate, and therefore in the dosage and in the therapeutic effect, was not due to the formation of a fibrous capsule around the pellets as postu-

lated by the authors, but was the consequence of the gradual reduction in the surface area.

For a patient requiring 4 mg. of testosterone propionate per day, a fairly constant absorption rate can be obtained by the following procedure. An initial introduction of 13 pellets of 50 mg. will give an initial absorption rate of 4.7 mg. The addition of 4 pellets after 70 days and every 60 days thereafter will yield a mean absorption rate of 4 mg. per day with a maximum fluctuation between 4.7 and 3.3 mg. per day, as illustrated in Fig. 7.

Pellets weighing 200 mg. have a tendency to slough out (Escamilla and Lisser, 1941; Howard and Jewett, 1942). This is not encountered

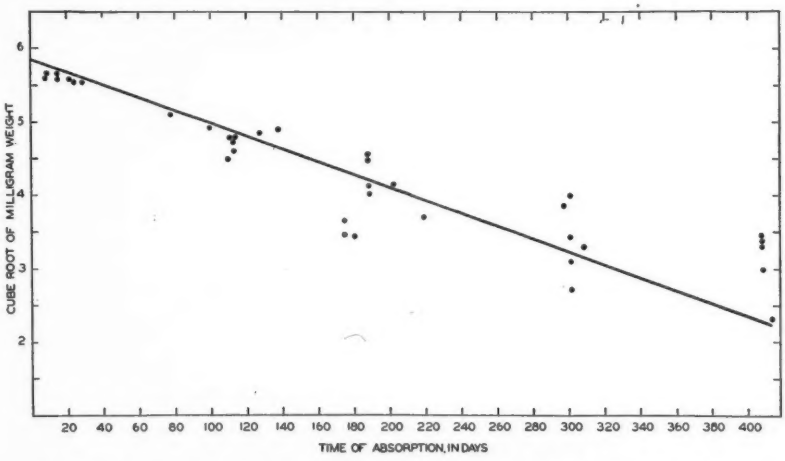

FIG. 6. Absorption of subcutaneously implanted pellets of testosterone propionate in man. Average weight of pellets, 202.6 mg.; dimensions, 6.5×6.5 mm. From data of Howard and Jewett.

with the use of smaller pellets (Biskind *et al.*, 1941; Shimkin and Zon, 1943). The smaller pellets have the additional advantage of simple implantation technic, requiring an ordinary paracentesis trocar, introduced subcutaneously after a small skin incision that is closed by a heated strip of tape. Figures 5 and 7 are merely illustrations and any size pellet ($h = 2r$) can be used and the desired dosage can be calculated if the constant c is known.

Thorn and his associates (1939, 1942) have shown that additional pellets of desoxycorticosterone acetate must not be implanted arbitrarily in Addison's disease, and that such additions should be based on the re-evaluation of the patient's requirements. This holds equally for the use of other hormones for protracted periods. With the correct assay of the needs of the patient, and with the application of the

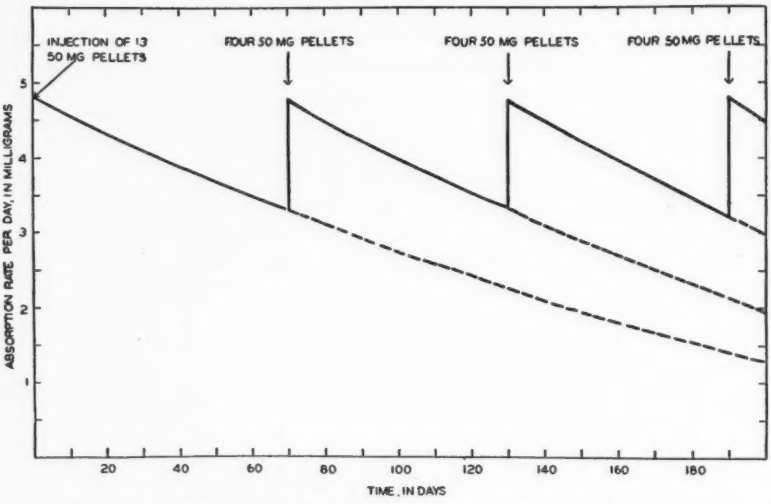

FIG. 7. Repeated implantations of testosterone propionate pellets as a means of establishing a continuous absorption rate of 4 mg. per day, in man. From data of Howard and Jewett.

above equations governing the absorption of subcutaneously implanted pellets of hormones, there are many interesting applications of this technic to clinical problems.

CONCLUSIONS

The decrease in rate of absorption of subcutaneously implanted pellets of steroid hormones is due to the change of surface area which depends upon the change in volume. This relationship is dependent upon the shape of the pellet. Control over the rate of absorption can be achieved by the application of these principles. In order to maintain an approximately constant level of dosage for a protracted period of time, it is necessary to implant additional pellets at regular intervals.

The validity of these postulations is demonstrated in mice by the study of the absorption of cylindrical pellets in which the diameter equals the length, and supported by the analysis of data on the clinical results with diethylstilbestrol and testosterone propionate.

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THE EFFECT OF THYROXIN INJECTIONS ON THE THYROTROPHIN CONTENT OF THE ANTERIOR PITUITARY OF THE MALE ALBINO MOUSE[1]

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THE ANALYSIS of the factors involved in the regulation of the secretion of the thyrotrophic hormone by the anterior pituitary has involved several types of investigations. Of these, the studies of thyroidectomy and of hyperthyroidism point to the thyroid hormone as the principal regulator of this pituitary hormone. On this hypothesis, a decrease in the level of thyroid hormone in the body should be associated with an increased secretion of thyrotrophic hormone and an increased supply of thyroid hormone with lessened production of thyrotrophin. The first part of this hypothesis is supported by the work of several investigators (Aron, Van Caulaert and Stahl, 1931; Ch'en and VanDyke, 1934; Bodart and Fellinger, 1936; Fellinger, 1936; Hertz and Oastler, 1936; Zeckwer, 1936; Emerson and Cutting, 1938; Rawson and Starr, 1938; Means, 1939; Starr *et al.*, 1939; Bastenie, 1940; Rawson, Sterne and Aub, 1942) who report a greater thyrotrophic potency of the pituitaries and body fluids of thyroidectomized dogs, rats and rabbits and of myxedematous and thyroidectomized human beings, but not by that of others (Houssay, Novelli and Sammartino, 1932; Hohlweg and Junkmann, 1933; Cope, 1938; Reforzo-Membrives, 1938; Reineke, Bergman and Turner, 1941) who find no such increase in thyroidectomized rats and goats or in cases of human myxedema. Turner and Cupps (1940) in fact claim a sex difference in the response of rats, the males showing a decrease of 46 per cent in thyrotrophic potency at 40 days after thyroid removal and of 50 per cent at 6 months, whereas at the end of the same periods, the females show a slight increase of 24 per cent or no change at all. The second part of the hypothesis is based on more consistent evidence. Administration of thyroid substance has reduced or eliminated the thyrotrophic potency of pituitaries and of urine in rats and guinea pigs (Aron, Van Caulaert and Stahl, 1931; Hohlweg and Junkmann, 1933; Kuschinsky, 1933; Franck, 1936; Reforzo-Membrives, 1938, 1940, 1942, 1943) and in hyperthyroidism in man tests of urine or blood indicate a lowered content or absence of thyrotrophin (Aron, Van

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Caulaert and Stahl, 1931; Krogh and Okkels, 1933; Bodart and Fellinger, 1936; Fellinger, 1936; Rawson and Starr, 1938; Rawson, Sterne and Aub, 1942). It has even been suggested by recent experiments on rats (Reforzo-Membrives, 1940, 1942, 1943) that excessive hyperthyroidism may induce the development of a thyroid-inhibiting factor in the anterior pituitary as well as the elimination of the thyroid-stimulating hormone.

In line with the above experiments, a study of the effect of the administration of thyroxin on the thyrotrophic potency of the anterior pituitary of adult male albino mice was undertaken, males being chosen because of previous experiments on mice of this sex (Adams and Allen, 1942; Adams and Beeman, 1942). The assay used was that employed by Adams and Beeman (1942), namely, the histological response of the thyroid of the day-old chick twenty-four hours after implantation of a single pituitary, since this assay has been shown to reveal the thyrotrophin present in very small amounts of fresh pars anterior.

MATERIALS AND METHODS

The albino mice used in the experiments were mature males, averaging 24 gms. in weight, secured from R. E. Pottorff of Littlestown, Pennsylvania. Three experiments were run, the first two containing three different series of thyroxin-treated mice and two series of controls, and the third a single thyroxin-injected series in addition to the control mice. The daily doses of Squibb's thyroxin used in the three series were as follows: series A (13 mice), 0.03 mg. in 0.3 cc. of distilled water; series B (8 mice), 0.06 mg. in 0.3 cc.; and series C (7 mice), 0.12 mg. in 0.3 cc. The injections were continued for 21 days in Experiments I and III, but in Experiment II injections were given for 23 days. The average total dose of thyroxin for series A was 0.64 mg.; for series B, 1.32 mg.; for series C, 2.66 mg. Two types of controls were run with each experiment: normal untreated mice and injected controls which received daily injections of 0.3 cc. of a solution of three drops of 4 per cent NaOH in 30 cc. of distilled water comparable to the solution in which the thyroxin was dissolved (table 1).

The day-old chicks used for assay were either a cross between a Rhode Island Red male and a Barred Rock female (Experiments I and III) or Rhode Island Reds (Experiment II), secured from Pilch Poultry Farms, Hazardville, Connecticut. After the mouse was killed, its pars anterior was removed, weighed and placed just beneath the skin of the chick's breast. The opening was sealed with 'New Skin.' (For fuller description of technique, see Adams and Beeman, 1942.) The thyroids of the mouse were then dissected out, weighed and fixed in Helly's solution.

Twenty-four hours after the mouse pars anterior had been implanted, the chick's thyroids were removed, weighed separately and fixed in Helly's solution. The sex of the chick was determined by gross inspection of the gonads. In each experiment, there were two types of chick controls: 1) two-day-old untreated chicks killed when the chicks implanted with anterior pituitaries were autopsied, and 2) controls which received varying amounts of fresh brain from the thyroxin-injected mice and were killed twenty-four

hours after its implantation. In addition, some day-old chicks were killed when implants were made (table 2). The mouse and chick thyroids were sectioned at 5μ, stained in Delafield's hematoxylin and counterstained in Mallory's connective tissue stain. Cell measurements were made at a magnification of 970 diameters with a Leitz ocular micrometer. In the chick thyroids, two cells were measured on the opposite sides of each of fifty follicles distributed across the diameter of the gland in one of the central sections.

TABLE 1. DATA CONCERNING MALE ALBINO MICE INJECTED WITH THYROXIN FOR 21 TO 23 DAYS AND CONTROLS (UNTREATED OR INJECTED WITH DILUTE NaOH) WHOSE ANTERIOR PITUITARIES WERE TESTED FOR THYROTROPHIC POTENCY

Mouse series	Number of mice	Average body weight gms.		Increase in body wt. dur. exper.		Average anterior pituitary weight		Average thyroid weight		Average thyroid cell ht. μ
		Initial	Autopsy	gms.	%	Abs. mg.	Rel. %	Abs. mg.	Rel. %	
THYROXIN-TREATED MICE										
Series A 0.64 mg. thyroxin	13	25.7	29.3	3.6	14.01	1.29	0.0043	4.40	0.0149	3.05±0.02
Series B 1.32 mg. thyroxin	8	21.6	23.7	2.1	9.72	1.13	0.0049	3.59	0.0156	3.01±0.02
Series C 2.66 mg. thyroxin	7	21.2	24.4	3.2	15.09	1.28	0.0053	3.72	0.0155	2.85±0.02
AVERAGE*	28	23.4	26.5	3.1	13.25	1.24	0.0047	4.00	0.0153	2.99±0.01
CONTROL MICE										
Untreated normals	9	24.4	28.7	4.3	17.62	1.40	0.0049	4.23	0.0146	5.60±0.03
NaOH-injected	9	24.7	28.6	3.9	15.79	1.49	0.0052	3.82	0.0135	4.89±0.03
AVERAGE†	18	24.6	28.6	4.0	16.26	1.45	0.0050	4.03	0.0141	5.25±0.02

* Average of individual data for all thyroxin-injected mice in series A, B and C.
† Average of individual data for all control mice (untreated and NaOH-injected).

In the mouse, it was necessary to include two central sections chosen at least five sections apart in order to secure fifty follicles suitable for measurement.

RESULTS

The effect of thyroxin injections in the mouse. The average increase in body weight for the three thyroxin-injected series A, B and C was 3.1 gm., or 13.25 per cent, which was 22.5 per cent less than that (4.0 gm.) of both control series (table 1). The fact that no loss in weight occurred over the 21 or 23 day periods of treatment indicated that the average total doses of thyroxin administered for each series (0.64, 1.32 and 2.66 mg., respectively) did not inhibit completely the normal growth processes. The average absolute weight of the pars anterior of the three series of thyroxin-treated mice (1.24 mg.) showed a decrease of 0.21 mg., or 14.48 per cent, from that of the control mice, while the average relative weight of the anterior pituitaries of the treated mice decreased 6.00 per cent (table 1). In the three thyroxin-injected series of mice, the average absolute weight of the thyroids (4.00 mg.) was very similar to that of the controls (4.03 mg.), but due to the smaller increment in body weight during treatment in

the former, their average relative thyroid weight (0.0153 per cent) was larger than that of the controls (0.0141 per cent) (table 1). The most striking change occurred in the histology of the thyroids of the thyroxin-treated mice when compared with that in the controls. The

TABLE 2. DATA CONCERNING DAY-OLD CHICKS WHICH RECEIVED ANTERIOR PITUITARIES (AP) FROM THYROXIN-INJECTED MALE ALBINO MICE OR FROM CONTROL MICE (UNTREATED OR NaOH-INJECTED) OR WHICH WERE UNTREATED OR IMPLANTED WITH MOUSE BRAIN IN TESTS OF THYROTROPHIC POTENCY

Type of mouse AP implanted	Av. wt. of mouse AP implanted mg.	Number of chicks	Average body weight, gms.		Average thyroid weight		Average thyroid cell ht. μ
			1 day	2 days	Abs. mg.	Rel. %	
CHICKS IMPLANTED WITH APS OF THYROXIN-INJECTED MICE							
Series A 0.64 mg. thyroxin	1.29	13	36.4	37.6	2.73	0.0073	2.24 ± 0.01
Series B 1.32 mg. thyroxin	1.13	8	39.9	40.8	2.74	0.0067	2.30 ± 0.02
Series C 2.66 mg. thyroxin	1.28	7	38.7	38.9	3.10	0.0080	2.28 ± 0.01
AVERAGE*	1.24	28	38.0	38.9	2.83	0.0073 ± 0.0002	2.26 ± 0.01
CHICKS IMPLANTED WITH APS OF CONTROL MICE							
Untreated	1.40	9	37.7	38.8	3.39	0.0088	3.63 ± 0.02
NaOH-injected	1.49	9	37.8	38.8	3.38	0.0086	3.68 ± 0.02
AVERAGE†	1.45	18	37.8	38.8	3.39	0.0087 ± 0.0006	3.66 ± 0.02
CHICK CONTROLS (2-DAYS-OLD)							
Mouse brain-implanted	Mg. brain implanted	6	35.7	35.2	2.86	0.0084	2.23 ± 0.02
Untreated	1.87	7		38.7	2.81	0.0074	2.09 ± 0.01
AVERAGE‡		13		37.1	2.84	0.0079 ± 0.0003	2.15 ± 0.01
NORMAL CHICKS (1-DAY-OLD)		7	37.2		3.15	0.0086	2.18 ± 0.02

* Average of individual data for all chicks implanted with APs from thyroxin-injected mice of series A, B and C.

† Average of individual data for all chicks implanted with APs from control mice (untreated or injected with dilute NaOH).

‡ Average of individual data for all 2-day-old chick controls (untreated or implanted with mouse brain).

follicles of the former were large, crowded against one another, filled with orange-staining colloid, and bounded by flattened epithelium whose average height for the three series was 2.99 ± 0.01 μ (table 1, fig. 1). In the controls the follicles were predominantly small, contained both blue- and orange-staining colloid, and their epithelial cells were cuboidal with prominent rounded nuclei. The average cell height of both sets of controls was 5.25 ± 0.02 μ (table 1, fig. 1). The difference between the average cell height of the thyroids of the thyroxin-treated mice and that in the control mice was 2.26 ± 0.02 μ, or 43.43 per cent, and was statistically significant. The average thyroid cell height of series C (2.85 ± 0.02 μ) which received an average total

dose of 2.66 mg. of thyroxin was also significantly less than those of series A (3.05 ± 0.02 μ) and series B (3.01 ± 0.02 μ) which were given average total doses of 0.64 and 1.32 mg. of thyroxin, respectively. The difference between the average cell heights of the thyroids of series B and series A was not significant.

The assay of the thyrotrophic potency of the mouse anterior pituitaries in day-old chicks. The average absolute thyroid weight (3.39 mg.) of those chicks receiving implants of pars anterior from the normal and injected mouse controls represented an increase of 0.55 mg.,

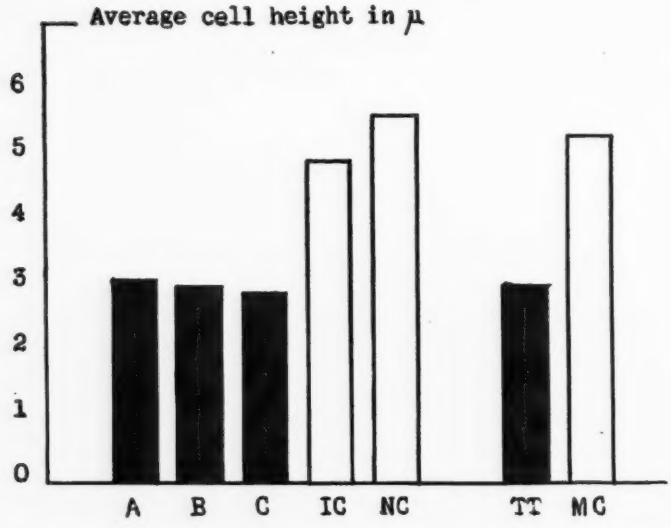

FIG. 1. Average cell height in thyroids of adult male albino mice injected with thyroxin (solid blocks) over 21 or 23 days. Mice in series A received an average total dose of 0.64 mg. of thyroxin; in series B, 1.32 mg.; in series C, 2.66 mg.; TT, all thyroxin-injected mice. Control mice (empty blocks) received weak NaOH solution (IC) or were untreated (NC); MC, all control mice. For data, see table 1.

or 19.37 per cent, over that (2.84 mg.) of the control chicks which were untreated or implanted with mouse brain (table 2). The average absolute weight of the thyroids of chicks implanted with anterior pituitaries of thyroxin-injected mice was 2.83 mg. or 0.35 per cent less than that of the controls. The relationships of relative thyroid weights in the groups were as follows: 1) an increase of 10.13 per cent in that of chicks receiving mouse control implants over that of the chick controls, and 2) a decrease of 7.59 per cent in those receiving pituitaries from thyroxin-injected mice compared with the controls. Neither of these figures was statistically significant. The thyroids of the chicks implanted with pars anterior from mouse controls (untreated or in-

jected with dilute NaOH) were very active compared with those of control chicks. The follicles were smaller, the colloid was predominantly blue and its borders were vacuolated. The cells were higher than those in controls, averaging 3.66 ± 0.02 μ after an average dose of 1.45 mg. of pars anterior compared with 2.15 ± 0.01 μ in the control chicks (untreated or brain-implanted) (table 2, fig. 2). The difference was an increase of 70.23 per cent and was significant. The frequency curve (fig. 3 A) showed a marked shift to the right compared with that of the controls. In contrast, the thyroids of the chicks implanted with anterior pituitaries of the thyroxin-injected mice were very similar in appearance to those of the chick controls. Even with an average dose of 1.24 mg. of pars anterior from the three thyroxin-treated series, the

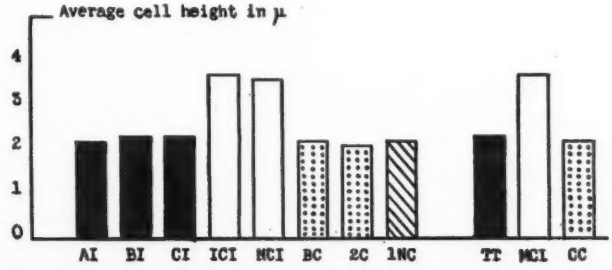

FIG. 2. Average thyroid cell height in series of chicks 24 hours after implantation of anterior pituitaries from thyroxin-injected mice of series A, B and C (solid blocks) or from control mice (empty blocks) injected with weak NaOH solution (ICI) or untreated (NCI). Two-day-old control chicks (stippled blocks) received implants of mouse brain (BC) or were untreated (2C). One-day-old chicks (barred block, 1NC) were untreated. TTI, all chicks with implants from thyroxin-injected mice; MCI, all chicks with implants from control mice; CC, all 2-day-old chick controls. For data, see table 2.

average thyroid cell height of the chicks was only 2.26 ± 0.01 μ (table 2, fig. 2). Nevertheless, the difference of 0.11 ± 0.02 μ, or 5.12 per cent, between this average figure and that of the controls (2.15 ± 0.01 μ) represented a significant increase. The incidence of the cell heights in the three series of chicks receiving pituitaries from thyroxin-injected mice when plotted nearly duplicated the curve for the controls (fig. 3 A). It was evident, too, (fig. 3 B) that the individual frequency curves of thyroid cell heights in the chicks given pituitaries from mice injected with different doses of thyroxin were similar.

DISCUSSION

The data obtained in the present experiment confirm for another mammal, the male albino mouse, the lessening of the thyrotrophic potency of the pars anterior of the thyroid-treated animal. In the light of the proven control of the thyroid by the pars anterior, this might be deduced from the condition of the follicular epithelium of the thy-

roids of the thyroxin-injected mice for there has been a significant reduction in thyroid cell height of 43.43 per cent in these animals compared with the height in normal mice (cf. *dog:* Kunde, 1927; Houssay,

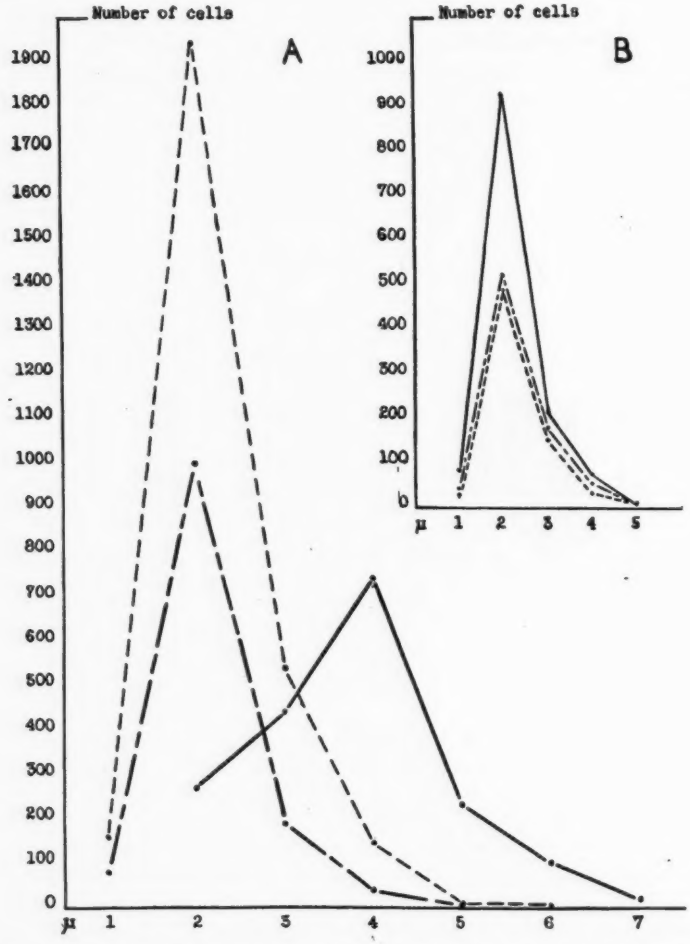

FIG. 3. A. Graph showing incidence of thyroid cell heights in chicks which received implants of anterior pituitaries from thyroxin-injected mice, — — —; or from control mice (untreated or NaOH injected), ——— ; and in 2-day-old chick controls which received implants of mouse brain or were untreated, — — — —. Measurements were grouped as follows: 1μ (0.9–1.5); 2μ (1.7–2.4); 3μ (2.6–3.4); 4 μ (3.6–4.3); 5 μ (4.5–5.5); 6 μ (5.6–6.4); 7 μ (6.6–7.5).

B. Graph showing incidence of thyroid cell heights in chicks which received implants of anterior pituitaries from mice injected with average total doses of thyroxin of 0.64 mg. (series A), ——— ; 1.32 mg. (series B), — — — ; 2.66 mg. (series C), – – – – – –over 21 or 23 days. Grouping of measurements as in 3 A.

Biasotti and Magdalena, 1932; *rat:* Cameron and Carmichael, 1921; Cameron and Sedziak, 1921; Abel, Backus, Bourquin and Gerard, 1925; Uotila, 1940; *mouse:* Adams, Medlicott and Hopkins, 1942). It is, however, definitely established when the anterior pituitaries of the mice are assayed by measuring the thyroid cell height in the day-old chick 24 hours after their implantation. An average dose of 1.45 mg. of pars anterior of normal male mice produced an increase of 70.23 per cent[2] in thyroid cell height compared with that in control chicks, while one of 1.24 mg. of anterior pituitary from mice injected with an average total dose of 1.34 mg. of thyroxin over 21 to 23 days gave an increase of only 5.12 per cent. A comparison of these two figures indicates a reduction of 92.71 per cent in the thyrotrophic potency. However, since different amounts of anterior pituitary substance were used, it is advisable to make the comparison in terms of 'chick units' per gram of fresh pars anterior. On the basis of similar tests in day-old chicks of the New Hampshire breed, Adams and Beeman (1942, p. 140) defined "a unit of thyrotropic hormone as that contained in an amount of fresh substance which will give a 100 per cent increase in thyroid cell height of five to ten 1-day-old chicks 24 hours after subcutaneous administration in a single dose." If this unit is used, the normal mouse pars anterior would contain 484 chick units per gm. of fresh tissue and that of the thyroxin-treated mice only 42 chick units per gm. The latter figure represents a decrease of 91.32 per cent, very similar to that (92.71 per cent) computed for percentage changes in cell heights, but more accurate in that it takes into consideration the relation between the amount of pituitary implanted and the response secured.

These results are somewhat similar to those secured for rats made hyperthyroid by Hohlweg and Junkmann (1933), Kuschinsky (1933) and Reforzo-Membrives (1938, 1940, 1942, 1943). However, their assays were made on the thyroid of the guinea pig, either by deter-

[2] The percentage increase of 70.23 per cent in average thyroid cell height elicited by the implantation of the control mouse pituitaries into day-old chicks of a Rhode Island Red X Barred Rock cross (or Rhode Island Reds) is less than that (138.6 per cent) which Adams and Beeman (1942) secured with day-old New Hampshires in their assay of normal male mouse pituitaries of the same strain (Series 2F, table 2, Adams and Beeman, 1942). This is due primarily to the lower initial thyroid cell height of the New Hampshires (New Hampshires: one-day old, average 1.631 μ; 2-day-old controls, 1.750 μ; Rhode Island Red X Barred Rock cross or Rhode Island Reds: one-day-old, 2.18 μ; 2-day-old controls, i.e., untreated and brain-implanted, 2.15μ) for in actual cell height measurements, the increase in the New Hampshires is only 1.397 μ compared with 1.51 μ in the other breeds. Bates, Riddle and Lahr (1941) have also reported a breed difference in the response of the thyroids of White Leghorns to anterior pituitary stimulation. The crosses used in this experiment also show a greater variation than was found in the New Hampshires. For example, the average cell height for the 2-day-old untreated control chicks was 2.09 μ, that for 2-day-old brain-implanted controls 2.23 μ, which represents a 6.69 per cent difference in height, as much as occurred in chicks receiving anterior pituitaries from thyroxin-treated mice of series B and C. Furthermore, the 1-day-old chick controls in the present experiments have cells 2.18 μ in height or 4.31 per cent higher than the 2-day-old untreated controls (see table 2).

minations of weight or measurements of thyroid cell height, usually secured on the third or fourth days after two or three daily administrations of pituitary substance and the total doses (and number of pituitaries) administered and the periods of treatment were different. Hohlweg and Junkmann (1933) administered 0.025 mg. of thyroxin daily for 14 days to 30- to 40-gm. rats and estimated that the treatment reduced the thyrotrophin of the pars anterior 75 per cent. Kuschinsky (1933) gave 0.35 mg. daily for 5 to 18 days to 100-gm. rats and found that after total doses of 1.75 and 2.10 mg. in 5 and 6 days respectively, some thyrotrophin was still present, but that 4.20 to 6.30 mg. (12 to 18 days of treatment) completely eliminated it. In the present experiments, a relatively larger total dose (average, 1.34 mg.) of thyroxin per body weight (mouse, average body weight, 24.0 gm.) than that used by Hohlweg and Junkmann and also given a week longer reduced the thyrotrophin more than 90 per cent. However, when the three series are examined separately, it is evident that even though increased total dosages resulted in lowering the mouse thyroid cell heights, they did not result in further decrease in the thyrotrophic potency of the mouse pituitary, as in Kuschinsky's experiments on rats, for with the three average total doses (0.64, 1.32 and 2.66 mg. of thyroxin) employed in the series A, B and C and in each series for 21 to 23 days, there were similar increases in chick thyroid cell height (0.09, 0.15 and 0.13 μ or 4.19, 6.98 and 6.05 per cent, respectively,) over the average control figure (2.15 μ) when the anterior pituitaries were implanted. It is possible that the level obtained with the lowest dose represents a plateau in the descending curve of the thyrotrophin which cannot be overcome by injections of thyroxin two and four times as large. Therefore, from these results, a correlation of the quantity of thyrotrophin in the anterior pituitaries of thyroid-treated animals with doses of thyroxin administered would appear to be possible only after a shorter interval of treatment with thyroxin.

Recently Reforzo-Membrives (1938, 1940, 1942, 1943) has reported not only a reduction of thyrotrophin in the anterior pituitaries of thyroid-treated animals, but also the presence of a thyroid-inhibiting substance. He fed daily doses of 50 to 100 mg. of powdered thyroid for 22 to 45 days to normal or thyroidectomized adult male rats (weights between 150 and 200 gms.) and found that the anterior pituitaries of such rats caused a reduction of 26 per cent in the thyroid weight and thyroid cell height, of 26 to 48 per cent in basal metabolic rate, and of 23 per cent in the "oxidation index" of thyroid tissue of 150- to 200-gm. guinea pigs compared with the figures in untreated normal guinea pigs. On the basis of these results, he argues that the thyrotrophin has been completely eliminated from the anterior pituitaries and also that a thyroid-inhibiting or -depressing substance has been "originated or developed" in the hypophyses of the thyroid-fed

rats. No evidence of such an inhibiting substance has been secured in the present experiments, but neither has the thyrotrophin been completely absent from the pituitaries of the thyroxin-injected mice. It should also be emphasized that the doses of desiccated thyroid which according to Reforzo-Membrives produce the thyroid-inhibiting factor in the pituitary are relatively enormous. In his 1943 paper, he states that 50 mg. daily for three weeks, followed by 100 mg. daily for the fourth week, a total dose of 1,750 mg., is the most adequate treatment for the development of the thyroid-depressing substance in rats weighing between 150 and 200 gms. If one uses an average body weight of 175 gm. for the rat, a comparable dose for a 24-gm. mouse would be 240 mg. If the activity of the desiccated thyroid is estimated in terms of its total organic iodine (Lerman and Salter, 1934) which according to the U.S.P. should be 0.2 per cent, then the 1,750 mg. of desiccated thyroid should approximate the activity of 3.5 mg. of thyroxin and 240 mg. that of 0.48 mg. On the basis of a daily thyroxin requirement of 0.005 mg. for the rat, the amount necessary to restore normal basal metabolic rate in the thyroidectomized animal (Evans, Simpson and Pencharz, 1939), the dose given by Reforzo-Membrives for the first three weeks is twenty times and during the fourth week forty times the daily requirement. The present authors have not found any statement as to the daily thyroxin requirement of the normal albino mouse. In experiments on dwarf mice, Boettiger (1941) reports that "older dwarf mice given 8γ of thyroxin per day by mouth have an oxygen consumption similar to that of normal mice" (p. 792) and also that dwarfs tolerate even larger doses of thyroxin "in striking contrast to the sensitivity of normal mice to thyroxin after the removal of the thyroid" (p. 788) which suggests a daily requirement lower than 8γ for the normal mouse. The daily doses actually given in the present experiment, therefore, are probably considerably in excess of the normal daily requirement for the mouse, especially since they were injected, but even with these doses, the mice gained weight though to a smaller degree than the controls.[3] It is interesting in this connection that Koger, Hurst and Turner (1942) have found that daily doses of 0.04 and 0.02 mg. of thyroxin given to female albino mice of approximately 16-gm. average body weight (probably younger than the males used in the present work) for five weeks actually produced greater growth increments than those occurring in untreated controls during the same period. While it is possible that albino mice can be given doses of thyroxin which will 'originate or develop an active-thyroid inhibiting action in the hypophysis' in addition to directly inhibiting the activity of the thyroid and the thyroid-stimulating action of the anterior lobe as outlined for the rat by Reforzo-Membrives

[3] Reforzo-Membrives does not state whether losses or gains in body weight occurred in his thyroid-fed rats.

(1943), on the other hand, the mouse may show as specific a reaction in this respect as it does in resisting acetonitrile poisoning after thyroid administration, while the rat does not (Hunt, 1922–23).

The possibility of the presence of a thyroid-inhibiting factor in the anterior pituitary as well as a thyroid-stimulating one has been suggested by other investigators, notably by Smith (1931), but neither he (1942) nor Collip (1942) mentions its existence in their recent reviews of anterior pituitary hormones. While the experiments of Reforzo-Membrives (1938, 1940, 1942, 1943) furnish the most convincing evidence of the possible presence of a thyroid-inhibiting factor in the anterior pituitaries of markedly hyperthyroid rats, they do not contribute any indication of the existence of such a substance in the pars anterior of the normal animal, which might act in the control of the interrelationships of the thyroid and anterior pituitary. The bulk of the data appears to favor the hypothesis that there is a balance between the secretion of the thyrotrophin by the pars anterior and of thyroxin by the thyroid. This idea was suggested by Aron (1930), Loeb, Bassett and Friedman (1930) and confirmed by Houssay, Biasotti and Magdalena (1932), Van Eck (1939) and Uotila (1940), on the basis of experiments in which the thyroid-stimulating activity of the anterior pituitary was partially or entirely inhibited by simultaneously administered thyroid substance. It has received further support from the more recent experiments of Galli-Mainini (1941) and Rawson, Sterne and Aub (1942). In the experiments of Galli-Mainini, the increased oxygen-consumption of isolated sections of the guinea pig thyroid *in vitro* in human serum to which thyrotrophin had been added was nullified by the addition of thyroglobulin, and the decreased oxygen-consumption in the presence of thyroglobulin alone was likewise nullified by the addition of thyrotrophin. Similarly, in the work of Rawson *et al.*, the rabbit thyroid *in vitro* neutralized the potency of given amounts of thyrotrophin added to the medium and subsequently tested in the guinea pig. Thus the normal thyroid-anterior pituitary relationship might be pictured (see Galli-Mainini, 1941, fig. 3) as an interaction of the two hormones, thyroxin and thyrotrophin. A low thyroxin level in the blood would affect the anterior pituitary so that the latter's cells would produce more thyrotrophin and also a relatively inactive thyroid which would be more sensitive to thyroid-stimulating substance. In extremes of low thyroxin-production as in hypothyroid animals or after thyroidectomy, both the pituitaries and the body fluids have been reported to contain increased amounts of thyrotrophin, although there is some contradictory evidence on this point. A high thyroxin-level in the blood would affect the pars anterior so that its cells would produce less thyrotrophin or it might even neutralize directly the thyrotrophin produced (Van Eck, 1939) and also it would make the relatively active thyroid cells less sensitive to thyroid-stimulating substances. In experimental or

pathological hyperthyroidism, anterior pituitaries and body fluids should contain little or no thyrotrophin and the evidence in favor of this view is supplied by the experiments of previous investigators and by the present series.

SUMMARY

Three series of adult male albino mice were injected with average total doses of 0.64, 1.32 and 2.66 mg. of thyroxin over periods of 21 and 23 days. The degree of hyperthyroidism produced by 1.34 mg., the average dose for the three series, was accompanied by a smaller body weight increase than that in controls, though growth was not stopped, by a lessened activity of the thyroids of the injected animals amounting to as much as 43.43 per cent as judged by measurements of the follicular epithelium in the treated and control animals, and by decreases amounting to 14.48 per cent in the absolute weight of the pars anterior and to 6.00 per cent in its relative weight compared with the figures in the controls.

The thyrotrophic content of the anterior pituitaries of the thyroxin-injected mice and the control mice was assayed by implanting individual pituitaries subcutaneously into day-old chicks and determining the increase in their thyroid cell height over that of controls 24 hours after pituitary implantation. The average thyroid cell height of the chicks implanted with pituitaries of the three thyroxin-injected series was $2.26 \pm 0.01\ \mu$; that of chicks with pituitaries of control mice was $3.36 \pm 0.02\ \mu$; and that of control chicks (untreated or implanted with mouse brain) was $2.15 \pm 0.01\ \mu$. Thus the thyrotrophic potency of the pituitaries of the thyroxin-injected mice was decreased by 92.71 per cent compared with that of control mice. Furthermore, the average total dose of 0.64 mg. of thyroxin depleted the thyroid-stimulating content of the pars anterior as much as average total doses of 1.32 and 2.66 mg. given over the same period of time.

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EFFECT OF TESTOSTERONE PROPIONATE ON THE BODY WEIGHT AND SKELETAL SYSTEM OF HYPOPHYSECTOMIZED RATS

SYNERGISM WITH PITUITARY GROWTH HORMONE[1]

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BODY WEIGHT increase and skeletal growth have been reported in normal rats injected with testosterone. Some workers have only been able to demonstrate growth under limited conditions, and others have failed to demonstrate it. Age of animals, sex, dosage and length of treatment seem to have been some of the factors leading to the variable results. Rubinstein and Solomon (1940) found that small doses of testosterone propionate (50 μg. daily) stimulated the growth of normal and castrated male rats, whereas Rubinstein, Abarbanel and Kurland (1939) had earlier found that larger doses (1.0 mg. daily) depressed the growth rate. Shay, Gershon-Cohen, Paschkis and Fels (1941) found that testosterone in general had effects opposite to those of estrogen, that is, stimulated growth and depressed pituitary weight in normal female rats. No effect on growth was observed by them when male rats were injected from birth with 1 mg. three times weekly. Deansley and Parkes (1940–41) found that the body weight increase of normal female and male rats was inhibited to about the same extent by testosterone propionate and estrone (pellets). Turner, Lachman and Hellbaum (1941) found no effect of testosterone propionate up to a 2 mg. dose on body weight increase or bone growth in normal or castrated male rats, even with prolonged injection from the day of birth.

It has been commonly assumed that the effects of estrone on body weight were mediated through the pituitary. It is equally possible that the opposite effect of testosterone might also be mediated through the pituitary, perhaps by increasing the rate of growth hormone pro-

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duction, though it might equally well increase growth by synergism with the growth hormone produced at the normal rate. If the action of testosterone is thus through the pituitary, it should be easy to show that the hypophysectomized rat does not resume growth on the injection of testosterone. Body weight change has rarely been mentioned in papers dealing with tesotsterone injection in hypophysectomized rats. Leonard (1943), in a paper devoted to mammary stimulation of hypophysectomized male rats by testosterone, reported partial main-

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON BODY WEIGHT OF HYPOPHYSECTOMIZED MALE RATS

Group	No. of rats*	Age at hyph.	Days P.O. onset of injection	Daily dose	Days injected	Body weight, gm.			
						Hyph.	Onset	Autopsy	Change during injection
		da.		mg.					
Injected	3	40–41	0	1	15†	149		150	+ 1±8.7‡
Injected	6	40–41	0	2.5	15†	141		133	− 8±3.36
Controls	10	40–41		0	0	135		123	−12±2.2
Injected	4	60–74	0	1	15	277		250	−27±4.5
Controls	4	60–74		0	0	231		200	−31±5.2
Injected	5	91	0	1	16†	253		215	−38±7.5
Injected	4	91	0	4	16†	239		223	−16±3.2
Controls	5	91		0	0	223		199	−24±2.8
Injected	9	67–74	21–22	1	15	289	226	217	− 9±4.4
Injected	8	69–74	21–22	2	15	258	205	213	+ 8±6.1
Controls	12	67–74		0	0	255	202	193	− 9±3.2
Injected	6	29–31	14–15	1	20	86	85	94	+ 9±3.3
Controls	9	29–31		0	0	79	79	81	+ 3±1.3

* In this and all subsequent tables only data from completely hypophysectomized rats is included. Completeness of the operation was determined by observations made on the animal while alive, confirmed by examination of the sella turcica at autopsy, and finally, microscopic examination of any tissue arousing suspicion.

† Daily except Sunday, 13 injections in 15 days, or 16 days. The male sex hormone here and in subsequent experiments was injected subcutaneously in 0.1 to 0.2 cc. of sesame oil.

‡ The figures preceded by ± in this and subsequent tables are the Standard Errors.

tenance of body weight when he injected 10–12 mg. of testosterone over a 10 day period, whereas the controls lost weight.

Testosterone propionate in hypophysectomized male rats: 15 to 20 day injection period. Testosterone propionate[2] has been injected into a number of groups of hypophysectomized male rats in high daily doses (1 to 4 mg. subcutaneously in 0.1 to 0.2 cc. of sesame oil). The rats varied in age from 29 to 91 days at the time of hypophysectomy. Injections were started either on the day of operation or after a postoperative interval of 14 to 22 days, and were given daily for 15 or 20 day periods. The primary object of administration of large doses was maintenance or repair of the testes, but careful records were made of body weight changes. In these experiments, as will be seen in Table 1, no evidence was obtained for continuance of growth when injections were begun immediately postoperatively, or of re-establishment of growth after a postoperative period of growth stasis. It is true that the injected rats frequently lost less weight than their controls, but

[2] The testosterone propionate used in these experiments was kindly furnished us by Schering Corporation of New York through the courtesy of Dr. E. Schwenck.

this was not considered to be equivalent to growth. In a few cases the injected rats actually gained a small amount, but in no case did they gain a gram a day, the amount taken as the minimal significant response in the standardization of pituitary growth hormone, (or 1 R.U.).

Synergism of testosterone propionate with pituitary growth hormone

TABLE 2. GROWTH RESPONSE OF MALE RATS HYPOPHYSECTOMIZED AT 40 TO 41 DAYS OF AGE WHEN INJECTED FOR 20 DAYS FROM THE TIME OF OPERATION WITH TESTOSTERONE ALONE AND COMBINED WITH GROWTH HORMONE

SERIES I

No. of rats	Treatment		Body weight			Body length			Tibia	
	Substance	Daily dose*	Onset	Autopsy	Change	Onset	Autopsy	Increase	Length	Epiphyseal‡ cartilage
		mg.	gm.	gm.	gm.	cm.	cm.	cm.	cm.	micra
5	Testosterone propionate	0.25	139	123	−16±6.0	31.1	31.6	0.5±0.07	3.30±0.03	112± 6.7
4	Growth† Hormone	0.12	141	156	+15±4.9	30.9	32.5	1.6±0.02	3.38±0.03	280±13.0
5	Combination	0.25 0.12	143	171	+28±7.6	31.3	34.4	3.1±0.13	3.50±0.05	251±19.9
4	Control	0	134	117	−17±2.1	30.1	31.1	1.0±0.07	3.23±0.07	103± 8.6
						SERIES II				
5	Testosterone Propionate	0.25	133	124	− 9±2.3	30.2	31.1	0.9±0.24	3.33±0.05	121± 9.3
6	Growth† Hormone	0.25	136	161	+25±7.6	30.6	33.1	2.5±0.25	3.49±0.05	267±31.2
6	Combination	0.25 0.25	137	183	+46±5.3	31.2	34.8	3.6±0.29	3.61±0.06	279±17.1
5	Control	0	134	118	−16±3.4	30.6	32.3	1.7±1.4	3.29±0.09	119±9.21

* The growth hormone was injected intraperitoneally, the testosterone propionate subcutaneously.
† The growth hormone used (pH XXXV) was a purified preparation which contained approximately 20 hypophysectomized rat units per mg. When tested in hypophysectomized rats for contaminants at a total dose of 5 mg. in 4 days, it was found negative for gonadotropic and adrenocorticotropic hormone, and showed only a trace of thyrotropic hormone.
‡ Uncalcified portion of the proximal epiphyseal cartilage measured after silver nitrate staining.

in hypophysectomized male rats, 20 day injection period. As Rubinstein and Solomon (1941) had found that small doses of testosterone (50 μg) stimulated growth in normal and castrated rats whereas large doses (1 mg.) depressed the growth rate, it was thought possible that the negative results in the experiments just reported were due to overdosage. Smaller doses were therefore injected to see if these would cause growth after hypophysectomy. In case these results too should be negative, growth hormone was administered with the testosterone to some groups. If growth hormone action were augmented by testosterone, a key to the mechanism of action in normal animals would be available.

Male rats were hypophysectomized at 40–41 days of age. They were distributed in groups of 5 or 6 and injections were started on the day of operation. One group in each of the two series received testosterone propionate in 0.25 mg. doses, in 0.1 cc. sesame oil, subcutaneously, daily for 20 days. A second group was given growth hormone intra-

peritoneally. (In Series I the average daily dose of growth hormone was 0.12 mg., and in Series II the daily dose was 0.25 mg.). A third group received both substances in the doses described above. The fourth group of rats was held as controls.

Table 2 shows that the rats receiving testosterone alone lost about as much weight as did their controls. The body length increments (nose to tail tip) scarcely equaled the slow growth of the controls. The dissected tibias were also measured and found to be equal in length to those of the controls. The uncalcified portion of the proximal epiphyseal cartilage of the tibias was still as narrow as that of the controls. In contrast with these negative findings, testosterone propionate did appear to have an effect on growth in the presence of growth hormone. The body weight increase of the groups receiving the combined therapy was greater than that caused by growth hormone alone. The body length increased more and the length of the tibia was greater. The uncalcified portion of the proximal epiphysis of the tibia was wider than in the controls though of the same width as in the groups receiving growth hormone alone. As the tibia was known to have increased more in length from the combined treatments it was assumed that an equilibrium between cartilage growth and erosion had been reached in the 20 day period of injection. It was thought that difference in cartilage proliferation might be detected by use of a shorter injection period (see later section).

The proximal ends of the tibias (of animals in Series II, Table 2) were cut and studied histologically. In the controls the cartilage of the disc was narrow, there was practically no vacuolation of cartilage cells and no new bone formation was occurring. Only a few coarse trabeculae of bone touched the cartilage, and in the wide areas between were marrow cells devoid of oriented capillaries. No new trabecular bone was being formed, and in some, bone formation was already beginning to seal the cartilage from the marrow. Study of this region in bones from testosterone-treated animals showed that the cartilage columns had been kept open by capillaries and that some new formation of bone was still occurring. (See later section dealing with more pronounced changes in female rats). Growth hormone injected animals possessed wide, active epiphyseal cartilages which were being rapidly eroded and replaced by bone, so that many delicate bony trabeculae extended into the marrow. No augmentation of these effects by the addition of testosterone to the growth hormone therapy could be detected.

Effect of testosterone propionate on growth of hypophysectomized immature female rats, 10 day injection period. As the growth responses to testosterone propionate have been reported to differ with the sex of the rat, female rats were also tested. The female rats were hypophysectomized at 26 to 29 days of age. In Series I, Table 3, a considerable period, 42 to 90 days, was allowed to elapse between hypophysectomy and the onset of the injection period. In Series II, the responses to

testosterone were compared after a short postoperative period (12 to 14 days, which is used in pituitary growth hormone tests) and after a longer postoperative period (75 to 101 days). The testosterone propionate was injected daily, subcutaneously, in 0.1 or 1 mg. doses for 10 days (9 injections). As will be seen in Table 3, the injected rats (especially those longer postoperative) did increase in body weight somewhat more than the controls, but less than is considered significant in testing response to pituitary growth hormone (1 R.U. requires a gain of 1 gm. daily). The tibias of the injected rats were no longer than those of the controls. The width of the proximal epiphyseal cartilage of the tibia was, however, significantly wider than in the controls.

Microscopic examination of stained sections of the heads of tibias from Series I confirmed the fact that bone growth had been stimu-

TABLE 3. GROWTH RESPONSE OF FEMALE RATS HYPOPHYSECTOMIZED AT 26 TO 29 DAYS OF AGE WHEN INJECTED FOR 10 DAYS WITH TESTOSTERONE PROPIONATE

SERIES I

No. of rats	Daily dose subc.	Onset period P. O.	Body weight			Tail length			Tibia	
			Onset*	Autopsy	Gain	Onset	Autopsy	Increase	Length	Epiphyseal† cartilage
	mg.	da.	gm.	gm.	gm.	cm.	cm.	cm.	cm.	micra
6	1.0	42 to 85	83	89	6				2.93±0.05	177±11.1
5	0.1	48 to 90	85	90	5				2.93±0.05	149± 7.3
4	0	42 to 49	83	85	2				2.96±0.18	112± 2.3
			SERIES II							
6	1.0	12 to 14	68	70	2	11.1	11.3	0.3		
8	0	12 to 14	69	68	−1	11.0	11.3	0.3		
8	1.0	75 to 101	90	96	6	12.2	12.4	0.2		
8	0	75 to 101	90	92	2	12.2	12.3	0.1		

* The body weights of rats in which a longer postoperative interval had elapsed before onset of this experiment were larger due to the fact that the rats had been injected previously in growth hormone tests. A period of 15 days or more had, however, elapsed since this initial use.

† Epiphyseal width measured in $AgNO_3$ stained split tibias.

lated. The vacuolated layer of the epiphyseal cartilage was much more conspicuous. Both levels of testosterone (1 and 0.1 mg.) had caused a definite increase in the number of capillaries directed against the cartilage. Formation of new trabeculae of bone was occurring. The strength of the stimulus to ossification, or the length of injection, was apparently inadequate to establish parallel arrangement of the trabeculae, as they were still disordered in their arrangement. (Plate I, Fig. 2). Examination of the proximal ends of the tibias from Series II confirmed the results of the first series. In rats injected with testosterone the vacuolated layer of cartilage cells was more distinct than in the controls, and capillary invasion and new bone formation were occurring. The phenomenon was more distinct in rats in which a longer postoperative period had elapsed before injection of testosterone was begun.

Effect of acute injection (4 days) of testosterone propionate in hypophysectomized 26 day old females. Synergism with pituitary growth hormone. An effort was made to see whether stimulation of the

PLATE 1
Stain H and E Magnification 130×

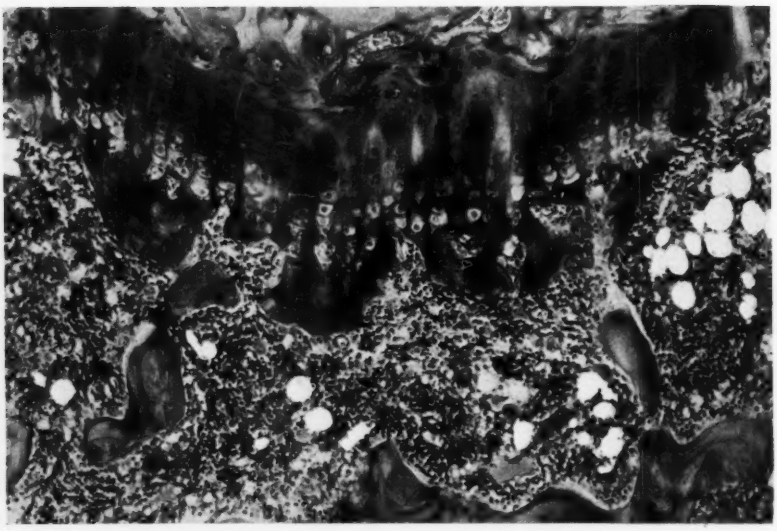

FIG. 1. Proximal epiphyseal region of tibia of uninjected control rat, female, 27 days old at hypophysectomy, and 59 days postoperative at autopsy. Spec. 8544. Pl. 8142

FIG. 2. Proximal epiphyseal region of tibia of female rat 28 days old at hypophysectomy, 48 days postoperative at onset of injection of testosterone propionate, 1 mg. daily, 9 injections in 10 days; 58 days postoperative at autopsy. Spec. 8533. Pl. 8141.

epiphyseal cartilage by testosterone could be detected by a more acute test (4 days). Reasoning from experiments with growth hormone (Kibrick, Becks, Marx and Evans, 1941), it was thought that the proliferation of the cartilage in this early period might be faster than its conversion to bone, and that a chondrogenic stimulus might thus be more readily detected than after a longer period of injection. Female rats 26 days old were hypophysectomized and were injected after a postoperative period of 13 days. As will be seen in Table 4 (Series I) no body weight increase above that of the controls occurred at either 1.0 or 0.05 mg.; daily doses of testosterone propionate. The

TABLE 4. EFFECT OF ACUTE INJECTION (4 DAYS) OF TESTOSTERONE PROPIONATE ON THE WIDTH OF THE EPIPHYSEAL CARTILAGE IN HYPOPHYSECTOMIZED 26 DAY OLD FEMALE RATS

Synergism with Pituitary Growth Hormone

SERIES I

No. of rats	Treatment		Body weight		Epiphyseal* cartilage
	Substance	Daily dose	Onset	Gain	
		mg	gm	gm	micra
8	Testosterone Propionate	1.00	68	2 ± 0.55	169 ± 11.6
8	Same	0.05	68	2 ± 0.86	168 ± 6.6
8	Control	0	67	1 ± 0.50	169 ± 5.0
		SERIES II			
7	Testosterone Propionate	0.25	68	0.4 ± 0.68	165 ± 4.1
7	Growth Hormone‡	0.10	68	8 ± 1.48	258 ± 14.5
7	Combination	0.25 0.10	68	8 ± 0.77	284 ± 12.8
7	Control	0	68	1 ± 0.81	163 ± 8.95

* Uncalcified portion of the proximal epiphysis of the tibia (silver nitrate stain).
‡ The growth hormone used was the same as used in previous experiments, see Table 2, footnote.

uncalcified portion of the proximal epiphyseal disc of the tibia also showed no increase in width. Examination of sections of the head of the tibia stained with hematoxylin and eosin, similarly disclosed no differences in the ossification process. The epiphyseal cartilage and zone of erosion were quiescent as in the hypophysectomized controls.

An attempt was also made to demonstrate a synergic action between testosterone and growth hormone within this short injection period. As will be seen in Series II of Table 4, the body weight and the width of the proximal epiphyseal disc of the tibia, were again the same in the group receiving testosterone as in the control. In the group receiving growth hormone, and the group receiving testosterone with the growth hormone, there was some increase in body weight, and an increase in the width of the epiphyseal cartilage, but the groups could not be distinguished by these criteria. Histological examination of the tibias of the animals receiving growth hormone, and those receiving the combined treatment showed great stimulation, and vigorous bone

formation, but no differences between the two groups could be detected. In the group receiving testosterone alone, and in the controls, the epiphyseal cartilage and the area of erosion were quiescent.

SUMMARY

In order to determine whether testosterone has a direct effect on body weight and skeletal growth, hypophysectomized rats, males and females, have been injected with testosterone propionate in daily doses ranging from 0.05 mg. to 4.0 mg. The effects in different age groups have also been tested (females, hypophysectomized at 26 days, and males, operated at 30, 40, 60 and 70 days of age). Injections have been given after different postoperative intervals, (0 to 100 days after hypophysectomy) and for different lengths of time (4 to 20 days). The following results have been obtained:

1. Testosterone propionate did not cause a significant increase in body weight or skeletal dimensions in hypophysectomized male rats. There was evidence, however, in male rats hypophysectomized at 40 days of age and injected with testosterone for 15 days following operation, that the cartilage columns continued to be eroded and to be replaced by bone. In other words, osteogenesis did not become quiescent in this period as it did in controls.

2. Body weight increase and increase in skeletal dimensions in hypophysectomized male rats injected with the pituitary growth hormone were augmented by the simultaneous injection of testosterone propionate.

3. Hypophysectomized female rats did not show significant body weight gain, nor tail length increase, nor epiphyseal width increase (tibia) when injected with testosterone propionate for a ten day period after a postoperative period of regression. However, some reactivation of epiphyseal cartilage and resumption of osteogeneis was noted at the proximal end of the tibia. These changes were not seen after a four day injection period.

That the undoubted initial effects of testosterone in awakening the osteogenic process would be indefinitely continued, leading to outspoken growth of hypophysectomized animals has not here been claimed.

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THE ESTROUS RABBIT AS A QUANTITATIVE ASSAY ANIMAL[1]

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In 1932 Friedman recommended the post-partum rabbit as a suitable animal for the quantitative assay of gonadotrophic substances, and seven years later (1939), he commented on the fact that only one group of investigators had published results based on this assay procedure. At this time he presented data on the sensitivity of the post-partum rabbit to chorionic gonadotrophins and their statistical significance for quantitative assays. However he pointed out that there was less regularity in the response of rabbits to pituitary extracts. During the past 3 years it has been necessary to use the post-partum rabbit in a continuation of a project started in this laboratory (Friedman and Mitchell 1941). Some of the limitations and fallacies of this rabbit assay method that have become obvious in the course of this work are presented in this communication.

In a previous report (Borasky and Bradbury, 1942) it was noted that post-partum estrous rabbits may ovulate following an injection of plant juice extracts, if they are treated during the spring and early summer months, but they rarely ovulate if treated during the fall and winter months. These results indicate that there is a seasonal variation in the sensitivity of the post-partum rabbit to plant juice extracts. Furthermore, it was found that there is a high incidence of anestrus during the winter months which is correlated with a definite loss in the weights of the pituitary, ovaries and uterus (Bradbury, 1944a). These findings made it necessary to determine whether there is a seasonal variation in the sensitivity of the estrous rabbit to pituitary extracts.

MATERIALS AND METHODS

In order to test the sensitivity of the rabbits at different times of the year it was necessary to have a supply of a stable pituitary powder. A sheep

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pituitary powder which had been prepared and tested in January, 1940, was re-tested in January, 1942, and found to have retained its potency. Twenty-four portions of this sheep preparation ranging from 20 to 30 mg. were weighed into ampoules, sealed in an atmosphere of nitrogen and then stored in the refrigerator. From time to time, one of these ampoules was opened and an extract prepared for assay. The extraction was made with dilute alkali (1 drop $N/10$ NaOH in 10 cc. water) and made up to a volume of 1 cc. per milligram of powder. These extracts were injected intravenously; in some instances the dosage was given on a body weight basis and in other instances the same absolute dose was given to a group of rabbits irrespective of their body weight.

The rabbits used in this study have all been purchased from the same dealer. For $2\frac{1}{2}$ years they were shipped to the laboratory when about 2 weeks pregnant and the litter was removed from the cage the day it was born. The rabbits were used for assay purposes during the first 3 weeks of the post-partum interval and subsequently after an induced pseudo-pregnancy. Non-pregnant rabbits have been purchased during the past year.

Since July, 1942, estrous rabbits, post-partum or otherwise, have been selected on the basis of abdominal palpation of the uterus. The uterus of the estrous rabbit is large, tonic and easily palpated. The uterus of the non-estrous rabbit is small and may not be palpable in an animal that is somewhat obese. The sexual condition of the rabbit was verified at the time of laparotomy. The estrous ovary is large and is made up of an opaque white matrix. The anestrous ovary is small and transparent or translucent and lacks the white opacity. As a result of injecting gonadotrophic extracts, ovaries which are not fully estrous may form hemorrhagic follicles, and cystic hyperemic follicles that subsequently luteinize without ovulation, and the rabbits experience a pseudo-pregnancy. For assay purposes these are considered positive responses in rabbits that are not fully estrous.

EXPERIMENTAL OBSERVATIONS

During the 12 months from June, 1942, through May, 1943, the incidence of anestrus was noted in 156 rabbits which had been laparotomized 2 to 4 weeks post-partum (table 1). In this series the

TABLE 1. INCIDENCE OF ANESTRUS IN POST-PARTUM RABBITS

Litters born	Rabbits having litters	Rabbits anestric 2 to 4 weeks post-partum	Per cent anestric
June–July 1942	26	10	38
August–September 1942	27	5	18
October–November 1942	36	18	50
December–January 1943	19	2	10
February–March 1943	31	2	6
April–May 1943	17	5	30

incidence of anestrus was lowest (6 per cent) in rabbits that had kindled in February and March, and highest (50 per cent) for those kindling in October and November. These figures compare favorably

with those given by Friedman for "isolated" non-parturient rabbits. The variations in weights of the ovaries, uterus and hypophysis of the estrous and anestrous domestic rabbits (Bradbury, 1944a) are similar to those found in the wild cotton-tail (Elder and Finerty, 1942). Friedman (1938) noted that the post-partum rabbit may become anestrous in the fall and winter months; and we have verified his finding that the anestrous rabbit is only one-fourth to one-fifth as sensitive as the estrous rabbit. The incidence of anestrus is such that it questions the contention, "that estrus occurs with great regularity in the post-partum animals" (Friedman, 1939).

Extracts made up from the reference powder were tested for their ovulation potency in rabbits at intervals from April, 1942 until

TABLE 2. INCIDENCE OF OVULATION IN ESTROUS RABBITS INJECTED WITH EXTRACTS OF A SHEEP PITUITARY POWDER AT DIFFERENT SEASONS OF THE YEAR

Test date	Dose in milligrams per kilogram of body weight												
	.10	.12	.14	.16	.18	.20	.22	.25	.30	.35	.40	.50	.75
4/29/42			3:4	4:1									
6/11			1:5	3:2	1:2								
7/21					2.2	3.0							
7/21	*anestric rabbits*				*0.3*	*0.3*	*0.3*						
8/11			0:5		0:5			0:1					
9/1									0:1		0:1	2:0	2:0
9/28									1:2		3:0	3:0	
11/7					0:2	5:0	1:0						
11/18		2:1			3:1			4:2					
12/3		1:4		3:2									
2/23/43	0:2		3:2		3:1		4:0						
5/15	0:1		0:4		1:1		2:1						
7/12				1:2			5:2			1:1			
8/17					1:0		3:0	1:0	1:0		1:0		
10/19								1:5		3:0	0:1	0:1	

The figures expressed as ratios indicate the number of rabbits responding positively (ovulation or luteinization) and the number failing to respond at each of the dose levels indicated. Example, 3:4 indicates that 7 rabbits were injected with the same dose per kilogram of body weight and of these, 3 ovulated and 4 were negative.

October, 1943, when the supply was exhausted. A summary of these tests is given in table 2. The most striking result is the high incidence of negative tests in August[3] and September, 1942, and again in October, 1943. During these months it required at least twice as much sheep pituitary extract to induce ovulation in rabbits, which were apparently in estrus, as was required in the spring months. These seasonal variations in the response of the test rabbits to pituitary extracts are similar to those noted for plant juice extracts.

In addition to this seasonal trend in sensitivity there is the individual variation in sensitivity which is of considerable magnitude. In

[3] These extracts were also assayed in immature female rats. The extracts prepared in August were just as effective in stimulating the infantile rat ovary as those extracts tested in April and June, 1942. These assays indicated that the negative responses of the rabbits in August were not due to a lack of potency of the extracts.

the test of July 12, 1943 (table 2) increasing the dose from 0.16 to 0.35 mg. per kilogram was not sufficient to give uniformly positive responses. Likewise on November 18 doubling the dose did not give any greater incidence of positive responses. This individual variation was also present in assays using rabbit pituitary extracts. (Table 3.) These tests show that, even when groups of 3 to 5 rabbits are used at each dose level, there is such a big difference between a dose which would give consistent positives and one which would give only negative results that quantitative estimations would be difficult within limits of 50 to 100 per cent. Similar results may have led Hill, Parkes

TABLE 3. TESTS WITH RABBIT PITUITARY EXTRACTS WHICH SHOW THE INDIVIDUAL VARIATION IN THE SENSITIVITY OF TEST RABBITS

	Dose in milligrams of fresh pituitary per kilogram of body weight				
3/13/43	0.75 negative negative positive		1.0 negative negative positive		1.5 negative positive
3/18/43	0.60 negative negative pseudopr. positive positive	0.80	0.9 negative pseudopr. pseudopr. positive positive	1.0	1.2 positive positive positive positive
Post-partum rabbits	pseudopr.	negative		positive	positive negative (anestric)

Pseudopr. denotes a rabbit that did not ovulate but in which there was luteinization of the unruptured follicles.

and White (1934) to recommend 20 rabbits at each dose level and caused other workers to discontinue using the rabbit.

In November, 1942, 13 post-partum rabbits and 12 rabbits which were judged estrous by abdominal palpation were all given the same absolute dose (0.75 mg.) of sheep pituitary extract. At the time of laparotomy, 5 of the 13 post-partum rabbits were found unsuitable for assay; 4 of these were anestrous and the fifth was already pseudopregnant from a spontaneous ovulation. Of the 8 remaining, 4 had ovulated, 2 had upruptured luteinizing follicles and the other 2 were negative. Of the 12 rabbits selected by abdominal palpation, 5 ovulated, 3 had unruptured luteinizing follicles and the remaining 4 were negative. Considered on a weight basis, there were positive responses at body weights of 4.6 and 5.3 kg. in the selected rabbits whereas the heaviest postpartum rabbit that responded weighed 3.9 kg. and 2 were negative at 4.4 and 4.5 kg. In the test on March 18, 1943 (table 3) one of the postpartum rabbits was anestrous and one was negative at a dose of 0.80 mg. whereas 2 selected rabbits had ovulated at a dose of 0.60 mg. per kilogram. These examples are representative of other

experiments in which it was found that when post-partum rabbits are selected on the basis of history alone, they are not as uniformly responsive nor as sensitive as isolated rabbits that are selected by abdominal palpation.

In some laboratories the estrous rabbit has been selected on the basis of a hypertrophy and hyperemia of the vulva (Leonard 1931, Smith, Severinghaus and Leonard 1933). During the routine examination of rabbits in the colony on April 28, 1944, 8 rabbits were found to have an estrous vulva but the uterus was judged small or anestric by palpation. Nine rabbits with estrous uteri and in the same body weight range were selected for comparison and all 17 were injected

TABLE 4. COMPARATIVE RABBIT-OVULATING POTENCY OF FOLLICLE STIMULATING HORMONE AND LUTEINIZING HORMONE

	Dose in milligrams per kilogram of body weight				
	0.003	0.006	0.012	0.024	0.050
L.H.	0:2	2:2	7:0		
F.S.H.			0:4	2:2	1:0

The figures expressed as ratios indicate the number of rabbits ovulating and the number failing to ovulate at each of the dose levels indicated.

with the same absolute dose (0.085 mg.) of follicle stimulating hormone. Only one of the group with a small uterus but an estrous vulva ovulated as a result of the injection and it was the smallest rabbit in the group (3.5 kg.). Of those selected with a palpably estrous uterus, 5 responded and the heaviest weighed 4.8 kg. This test indicated that it was better to judge the sexual condition of the rabbit on the basis of palpation of the uterus rather than on the appearance of the vulva. In many instances we have purposely included rabbits which were anestrous by palpation in a test group (See table 2, 7/21/42 and table 3, post-partum rabbit). Uniformly these anestrous animals have failed to ovulate even though the dose was double that which was effective in estrous rabbits.

In this study of the estrous rabbit it was of interest to determine the relative ovulating potency of the follicle stimulating hormone (F.S.H.) and the luteinizing hormone (L.H.).[4] For these two preparations it was found that a 0.006 mg. dose of L.H. was as effective as a 0.024 mg. of F.S.H. (table 4). These results indicate that the rabbit is more readily ovulated with L.H. than with F.S.H.

Testing unfractionated extracts such as the rabbit pituitaries (table

[4] The F.S.H. (Lot 43254) and the L.H. (Lot S-96) was supplied by Dr. J. P. Schooley, Difco Laboratories, Inc. According to their assays, the F.S.H. potency of the two powders (immature rat ovary) was 6 U./mg. for the F.S.H. and 1 U./mg. for the L.H. The L.H. potency of the two powders (rat seminal vesicle test) was 0.1 U./mg. for the F.S.H. and 3 U./mg. for the L.H.

3) in the estrous rabbit gave no indication whether the gonadotrophic hormone content was primarily L.H. or F.S.H. When these extracts were injected into immature rats, doses of 30 to 40 mg. were necessary to produce a definite increase in ovarian weight. When the rabbit and the rat assays are considered together it seems that the rabbit pituitary must contain more L.H. than F.S.H. These results confirm the suggestion of Hill (1934) that the discrepancy in assay values based on the response of the immature rat ovary and those based on the rabbit ovulation test is due to the fact that the rat ovary responds to F.S.H. while the rabbit ovulation is primarily a response to L.H.

DISCUSSION

The finding that 50 per cent of the rabbits may become anestrous immediately post-partum invalidates the supposed advantage of the post-partum rabbit for assay purposes. Since the uterus of the estrous rabbit is easily palpated, rabbits may be selected on the basis of their sexual condition at the time of assay. Rabbits selected on this basis are more consistent in their response than rabbits selected on the basis of past history or on the presence of a hypertrophied hyperemic vulva.

The high incidence of anestrus and the relatively refractory condition of the rabbits that appear estrous during the late summer and fall months indicates that the domestic rabbit still has a marked tendency to revert to the seasonal breeding condition of the wild rabbit. Further studies of optimal conditions of temperature, lighting, caging and diet may reveal how this seasonal variation in sexual condition may be minimized and the rabbit made a more standard test animal.

Hill, Parkes and White treated groups of rabbits at regular intervals and concluded that the rabbits became less responsive to a gonadotrophic substance after the third injection. It is unfortunate that their experiments were started in March and carried through into July and August, since it may be that the decreased responsiveness was due to the seasonal decrease in sensitivity rather than an immunity or foreign protein reaction. We have not attempted to keep groups of rabbits intact for periodic testing, but a few rabbits which failed to ovulate when given doses of .30 mg. of sheep pituitary during September and October did respond to .25 mg. in November and even smaller doses in February and March. Thus it is possible that Hill, Parkes and White might have obtained results suggestive of an increased sensitivity to repeated tests if they had started their experiments in August or September and carried them into April or May.

A similar interpretation is possible for the results of Friedman and Friedman (1939). Assays of rabbit pituitaries at intervals from September to May yielded results indicative of an increase in potency of the rabbit pituitary. In view of the fact that a reference powder will

also give an apparent increase in potency in successive tests from September to April, it seems more probable that their results were an expression of the seasonal increase in sensitivity of the rabbit, rather than any actual increase in hormone concentration in the pituitary. Elder and Finerty failed to find any seasonal variation in potency of the pituitary of the female cotton-tail rabbit when tested in the immature rat. Assays by the Friedman method have given higher gonadotrophic hormone values for beef pituitaries in the spring than in the fall (Friendman and Hall, 1941). Thus gonadotrophic assays based on the response of the estrous rabbit have no quantitative significance unless simultaneous comparisons are made using comparable groups of rabbits with at least 3 to 5 rabbits at each dose level. Furthermore, unless one of the substances used in a comparative assay is a standard reference powder it is not possible to judge the relative potency of a pituitary extract tested in the fall with that of an extract tested in the spring.

Ovulation is not a specific reaction in rabbits, since it may be induced by a number of substances and some of them are not gonadotrophic materials: copper (Brooks *et al.*, 1940), cadmium (Emmens 1940), picrotoxin (Marshall *et al.*, 1939) and plant juice extracts (Bradbury 1944b). This lack of specificity makes the rabbit ovulation test very inadequate as a qualitative procedure. If the nature of the substance being tested has been determined by chemical or differential rat assays, then the estrous rabbit may be used as a quantitative test animal. The data listed in this paper have been presented to show the limitations of the estrous rabbit for the assay of pituitary extracts. These limitations do not detract from the use of the rabbit as a qualitative and possibly a quantitative test for chorionic gonadotrophins.

SUMMARY

Post-partum rabbits may become anestrous in the first 4 weeks after kindling and the incidence of anestrus may be as high as 50% in the fall months. Estrous rabbits can be selected to best advantage by means of abdominal palpation of the uterus. Estrous rabbits, postpartum or otherwise, show a marked tendency toward a seasonal variation in sensitivity which detracts from their value for quantitative assays of pituitary extracts.

The individual variation in sensitivity of estrous rabbits to pituitary gonadotrophic extracts is so great that groups of rabbits must be used at each assay dose level if quantitative estimations within 50 to 100% are desired. The rabbit is more responsive to the luteinizing hormone than it is to the follicle stimulating hormone, but unless used in conjunction with rat assay procedures, the rabbit ovulation assays give no information as to the relative proportion of these two hormones in unfractionated pituitary extracts.

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CHEMICAL STUDIES ON MELANOGENESIS IN NORMAL AND ADRENALECTOMIZED RATS[1]

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THE INCREASE in pigmentation accompanying atrophy or tuberculosis of the adrenal cortex has been an established clinical fact since Addison's (1885) original description of the disease. Eiselt (1910) attributed the pigmentation to an increase in melanin and since then several investigators (Harrop 1933, Kelloway 1923, Konigstein 1910) have reported a slight to moderate degree of melanosis in animals following adrenalectomy. Recently work of this nature was reported in adrenalectomized black rats from this laboratory (Ralli and Graef 1943). In the study rats were fed a diet deficient in the filtrate factors of the vitamin B complex for a sufficient period of time before adrenalectomy to produce achromotrichia. Following adrenalectomy definite bluing of the skin occurred and on pathological section an increased deposition of pigment was observed in the hair follicles and bulbs, as well as a hyperplasia of the hair apparatus. The present report is a study of the chemical factors presumed to be involved in melanogenesis and supplements the histological and biological observations previously made (Ralli 1943). Among the factors involved in melanin formation are water content, particularly in relation to filtrate factor deficiency (Dyson 1911, Figge 1941); phenolic substances, generally considered as melanin precursors (Konigstein 1910, Kuwana 1940, Nobutani 1936); reduction and oxidation power of tissue, established by Figge as a fundamental influence on melanin formation from tyrosine (Figge 1940); tyrosinase activity identified in plants, bacteria, insects and amphybia (Raper 1928, Skinner 1942, Gortner 1911, Figge 1940) but not previously obtained from mammalian tissue; and melanin, the loosely classified pigment substance of mammalian skin (Gortner 1911). Because of occasional reports that melanin pigmentation is associated with lipid metabolism (Dyson 1911) and a general belief that the adrenal gland is concerned with lipid metabolism, fats and their degree of unsaturation were estimated in the skins of the animals. The chemical determinations were done on the hides of normal rats, nutritionally depigmented rats and rats that had been

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adrenalectomized and in which an increased deposition of pigment was present in the hair apparatus.

EXPERIMENTAL PROCEDURES

The tissue studied (shaved rat hide) was divided into 4 fractions: A, water soluble non-protein component containing precursors to melanin; B, water soluble protein component containing enzymatic activities associated with melanogenesis; C, tissue lipid; and D, melanin, the end product of melanogenesis. The fractionation is given in detail in flow sheet 1. *Solution A:* macerated rat hides were extracted initially with a saturated ammonium

FLOW SHEET I

TISSUE FRACTIONATION

Tissue—weighed, minced rat hide ground with sand (1–2 gram sample).
Extracted with water saturated with ammonium sulfate
(room temperature, $CHCl_3$ preservative)

Residue 1 ..... > *Solution A*
Water soluble non-protein fraction
Extracted with water adjusted to pH8 (McIlvaine's buffer)

Residue 2 ..... > *Solution B*
Enzyme containing protein fraction
Extracted with alcohol-chloroform (hot)

Residue 3 ..... > *Solution C*
Neutral organic solvent soluble
lipid fraction
Exhaustive hot extraction with 1% sodium hydroxide solution

Residue 4 ..... > *Solution D*
Alkali soluble melanin

Discard

sulphate solution in order to separate phenolic pigment precursors from the tissue. The acid pH established by hydrolysis of the ammonium sulphate aided solubility of the enzyme component (reported melanin forming enzymes are soluble in alkaline aqueous solutions but insoluble in acid). The high salt concentration inhibited solution of protein associated with the melanin complex. *Solution B:* extraction of Residue 1 with an aqueous solution buffered with McIlvaine's phosphate-citric acid buffer to pH 8.0 furnished an alkaline extraction medium as recommended for optimum solubility of tyrosinase (Raper 1928) and gave a protein containing solution which could be subsequently buffered to pH 6.4, optimum for demonstration of tyrosinase activity. This solution contained enzymatic activity capable of converting tyrosine into melanin. *Solution C:* extraction of Residue 2 with ethanol followed by warm alcohol-chloroform solution removed all material soluble in the neutral organic solvents. After drying solution C with anhydrous sodium sulphate, total lipid weight and lipid unsaturation were determined. Removal of the lipid at this stage prevented emulsification during the final extraction of melanin. *Solution D:* Residue 3 was extracted exhaus-

tively with hot 1% NaOH by the method recommended by Gortner (1911) for melanin. Many of the other extraction techniques were tried but none proved as satisfactory for maximal consistent yields of melanin.

For sampling, the skin of each experimental animal was treated separately. After shaving, the hide of the back was removed and dissected free from adhering subcutaneous tissue. Finely chopped samples of the hide were then weighed, macerated with quartz sand and extracted with the initial solvent. Chloroform served to prevent the growth of micro-organisms. 4 to 5 extractions with each solvent gave satisfactory separations. Each sample was treated according to the procedure outlined in the flow sheet and the various constituents estimated. Separate samples of skin were used for the estimation of the water content. Phenols (free) were determined colorimetrically by the method of Folin and Ciocalteu (1927). Results are expressed as mg. phenol per gm. tissue. Reducing power was estimated by the reaction of the initial extract with standard potassium permanganate solution. Aliquots of extract A were titrated directly with 0.01 N $KMnO_4$. The endpoint for this reaction was most sharply defined if the titration was carried out at 50–60°C. The reaction measured the extent to which the phenolic precursors could be oxidized by potassium permanganate. Results are expressed as milli-equivalents $KMnO_4$/gm. tissue. Tyrosinase-like activity was determined in the following manner. Solution B was adjusted to pH 6.4 by addition of the calculated amount of citric acid buffer containing dissolved tyrosine (1.5 mg./cc.). A trace of ferrous iron (as $FeCl_2$ solution) was added to catalyze the reaction (Veer 1939, Rothman 1940) and the solution incubated at 38°C. for 48 hours. After incubation the solution was made alkaline with NaOH to 1% effective concentration. This procedure inhibited the conversion of tyrosine to melanin, converted the leuco-melanin to melanin pigment and resulted in full development of the melanin color. Small amounts of ferric hydroxide and coagulated protein precipitated at this time were removed by filtration. The colored solution was compared with standard melanin. The melanin used for comparison was prepared from tyrosine by the above method, purified by acid precipitation and reprecipitation, then dried and weighed. For accurate color matching this standard was dissolved in 1% NaOH, as were the unknown solutions. In the absence of any tissue extract a small conversion of tyrosine to melanin occurred in the blanks with ferrous iron alone at pH 6.4 after incubation. All determinations were therefore controlled by blank analyses and the observed values corrected. Results are reported as mgm./gm. of tissue. When dihydroxy phenyl alanine (Dopa) was substituted for tyrosine in the procedure the blank readings were so high that tissue catalysis could not be estimated accurately and this procedure was therefore not included. Tissue lipids were determined gravimetrically on *Solution C* after filtering and drying. The iodine number was estimated by the method of Hanus. Melanin was obtained by exhaustive extraction with hot 1% NaOH. The extracted colored solution was compared colorimetrically with the known melanin standards previously described. Results are expressed as mgm./gm. of hide. Finely chopped samples of hide taken from the same lot as the tissue to be extracted, were dried at 105–110°C. to constant weight. The water content of the tissue was calculated by difference. The results are given in percentage.

Black rats of the Long-Evans strain, bred in the laboratory were fed the

diet deficient in the filtrate factors of vitamin B when 35 days of age and the rats that were adrenalectomized were operated after 28 to 30 days on the diet. The animals survived adrenalectomy for 12 to 15 days when maintained on the deficient diet. At the time of death they weighed from 70 to 100 grams and were about 70 to 75 days old. As the average weight of normal rats of this age in our colony was 160 grams, the weights of the adrenalectomized animals compared more favorably with normal rats of 37 to 47 days of age. The hides of the latter, as well as hides of 70 day old normal rats, were analyzed for the control values.

RESULTS

Normal rats. In table 1 the chemical constituents of the hides of the normal rats at 37, 47 and 70 days of age are given. Weight in

TABLE 1. NORMAL RATS (INDIVIDUAL DATA)

Age days	Weight grams	% H_2O skin	Phenol (free) Mg/Gm tissue	Reducing power Meq. $KMnO_4$/Gm tissue	Lipid		Tyrosinase MgM/Gm tissue	Melanin MgM/Gm tissue
					Gm/Gm tissue	Iodine #		
37	74		0.257	.046	.080	79		Lost
	71		0.290	.037	.150	69		22.8
	76		0.224	.028	.091	76		19.8
	83		0.165	.025	.089	61		21.2
	67		0.171	.024	.126	82		20.6
Avg.	74		0.222	.032	.107	73		21.1
47	117	61.8	0.214	.028	.063	48	41.8	25.0
	105	62.0	0.176	.029	.050	53	30.7	20.6
	120	62.8	0.228	.037	.035	55	26.8	29.7
	104	59.0	0.200	.030	.033	65	35.9	32.3
	92	59.8	0.194	.038	.013	61	29.3	30.1
	102	62.4	0.204	.031	.022	76	23.7	35.4
	103	59.9	0.228	.034	.026	87	39.9	21.6
	97	59.9	0.212	.030	.019	86	27.3	29.7
	98	61.5	0.204	.034	.016	78	30.9	37.1
Avg.	104	61.0	0.206	.032	.031	68	31.8	29.0
70	163	61.0	0.235	.037				
	146	65.6	0.181	.031				
	173	61.6	0.207	.030				
Avg.	161	62.7	0.207	.033				

grams, per cent water, free phenol content, reducing power, lipid, iodine number, tyrosinase activity and melanin content of the hides are reported. The values obtained were fairly consistent and did not show any marked variation due to age or weight of the rats except for the total lipid concentration. Here there was considerable variation probably reflecting the age or the nutritional status of the animal (3). The hides of the younger rats gave higher lipid values than did those of the older animals but the degree of lipid unsaturation, as reflected by the iodine number, was constant. Sex did not seem to influence the values obtained. Figure 1 shows graphically the individual data for each rat of the 47 day old group.

Adrenalectomized and non-adrenalectomized rats fed the diet deficient in the filtrate factors of vitamin B. In table 2 are summarized the data obtained on 12 rats that received the deficient diet supplemented with 1% NaCl in the drinking water. Three of the animals were fed

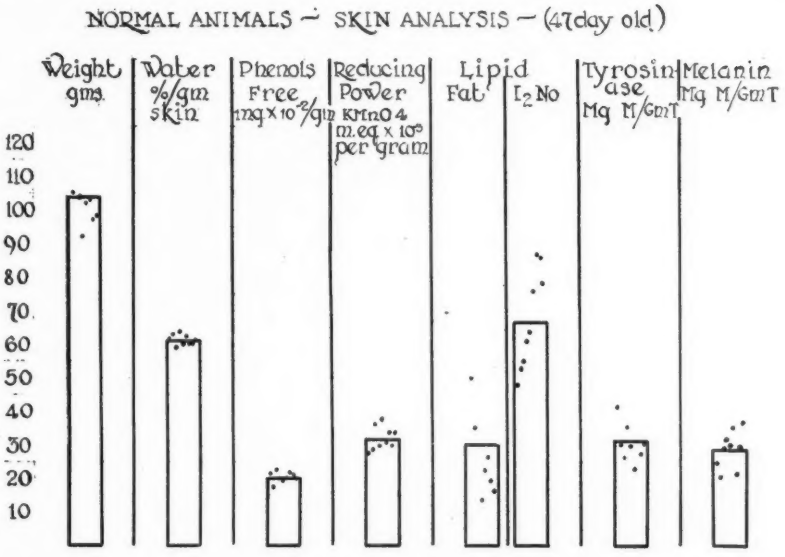

FIG. 1

the deficient diet for 40 days and the remaining 9 received the diet for 60 days. The most striking chemical changes occurred in the 9 animals that had received the diet for the period of 60 days. All of the animals developed marked graying of the fur. The phenol content of the hides was increased slightly above that of the normal rat. There was a definite decrease in the reducing power as reflected by the titration with $KMnO_4$. Tissue lipids were also significantly lowered but there was little change in the iodine number. The most outstanding effect of the deficient diet was the sharp reduction produced in the melanin content of the hide.

TABLE 2. FILTRATE FACTOR DEFICIENT RATS (INDIVIDUALS)

Def. diet days	Weight grams	% H_2O skin	Phenol (free) Mg/Gm tissue	Reducing power Meq. $KMnO_4$/Gm tissue	Lipid		Tyrosinase MgM/Gm tissue	Melanin MgM/Gm tissue
					Gm/Gm tissue	Iodine #		
40	85	68.0	0.200	0.016	.011	75		15.7
	62	65.3	0.223	0.013	.013	62		14.4
	71	69.7	0.188	0.010	.013	84		17.0
	77	67.5	0.203	0.013	.012	77		15.7
60	78	66.9	0.252	0.030	.018	75.0	22.1	10.0
	87	65.9	0.258	0.009	.021	81.5	24.0	22.9
	82	63.8	0.284	0.027	.012	61.7	33.6	10.7
	89	66.1	0.238	0.011	.013	81.5	32.3	13.4
	75	65.4	0.238	0.019	.025	70.8	39.6	12.2
	63	65.3	0.328	0.023	.017	67.9	43.0	15.2
	75	65.8	0.278	0.018	.013	59.4	43.9	14.6
	61	65.8	0.287	0.016	.014	50.8	28.4	12.9
	57	65.0	0.321	0.016	.015	62.5	37.2	19.5
	74	65.7	0.275	0.019	.017	67.9	33.8	14.6

The rats that were *adrenalectomized* were divided into 2 groups. Ten rats were fed the deficient diet for 28 days prior to adrenalectomy and 5 rats received the deficient diet for 45 days and were then adrenalectomized. Both groups received 1% NaCl solution ad lib before and after operation. All the animals showed increased deposition of pigment as evidenced by the blue color of the skin. The degree of pigmentation depended on the length of the post-operative survival (Ralli and Graef, 1943). The chemical analyses of the hides of the rats is recorded in Table 3. The preoperative duration of the deficient

TABLE 3. FILTRATE FACTOR DEFICIENCY—ADRENALECTOMIZED

Def. diet days pre-op.	Survival days	Weight grams	% H_2O skin	Phenol (free) Mg/Gm tissue	Reducing power Meq. $KMnO_4$/Gm tissue	Lipid		Tyrosinase MgM/Gm tissue	Melanin MgM/Gm T.
						Gm/Gm tissue	Iodine #		
28	13	82	70.6	0.447	.045	.015	43.5		33.2
	12	98	67.1	0.278	.027	.009	43.5		29.8
	8	91	69.6	0.226	.025	.014	46.0		26.3
	4	98	66.3	0.227	.019	.013	41.5		23.7
	5	88	68.1	0.248	.025	.013	43.5		24.1
	4	94	71.5	0.703	.042	.008	42.2		16.0
	4	100	66.5	0.686	.034	.007	42.5	25.6	22.0
	9	86	67.3	0.730	.046	.003	43.6	22.5	20.4
	12	112	68.7	0.676	.037	.005	34.6	37.6	20.6
	12	95	67.3	0.762	.026	.006	33.0		18.8
	8.4	94	68.3	0.498	.033	.009	41.2	28.6	23.5
45	7	93	69.4	0.403	.024	.010	30.0		19.7
	9	87	70.5	0.622	.049	.011	35.0		14.6
	10	109	68.0	0.424	.035	.007	47.0		18.3
	10	81	68.5	0.384	.039	.005	28.0		19.1
	15	88	67.8	0.513	.027	.009	38.8		17.7
	10.2	92	68.8	0.469	.035	.009	35.7		17.9

diet affected only the final melanin content of the skin, which was lowered in the rats that had received the deficient diet for the longer period. The melanin content was 23.5 mg/gm tissue in the rats receiving the deficient diet 28 days before operation and 17.9 mg/gm tissue in the 45 day deficient rats. Both of the values are higher than in the unoperated filtrate factor deficient groups (Table 2), but lower than the values (Table 1) obtained in the normal groups. The results on the normal, filtrate factor deficient and filtrate factor deficient-adrenalectomized rats are compared in Figure 2. Significant changes occurred in the water content, the free phenol content, the reducing power, the degree of lipid unsaturation and the extractable melanin of the hides. The filtrate factor deficient diet resulted in an increase in the water content of the skin of both operated and unoperated animals. The phenol contents of the skins of filtrate factor deficient animals rose slightly above normal. Adrenalectomy induced a marked upward shift. The reducing power of tissue extracts, as shown by $KMnO_4$ titration, was decreased in the filtrate factor deficient group, but there was a return to normal in this group following adrenalectomy. There was a diminution in the skin lipid accompanying filtrate factor deficiency but there was no significant change in the degree of lipid unsaturation as shown by the iodine number.

Adrenalectomy of the filtrate factor deficient animals resulted in a fall in iodine number. Tyrosinase activity was demonstrable in all cases. No significant variation was observed in the groups studied. Apparent differences were in all probability within the limits of experimental variation. Extracted melanin declined sharply but did not disappear entirely as a result of filtrate factor deficiency. It returned toward the normal values following adrenalectomy. This finding is a

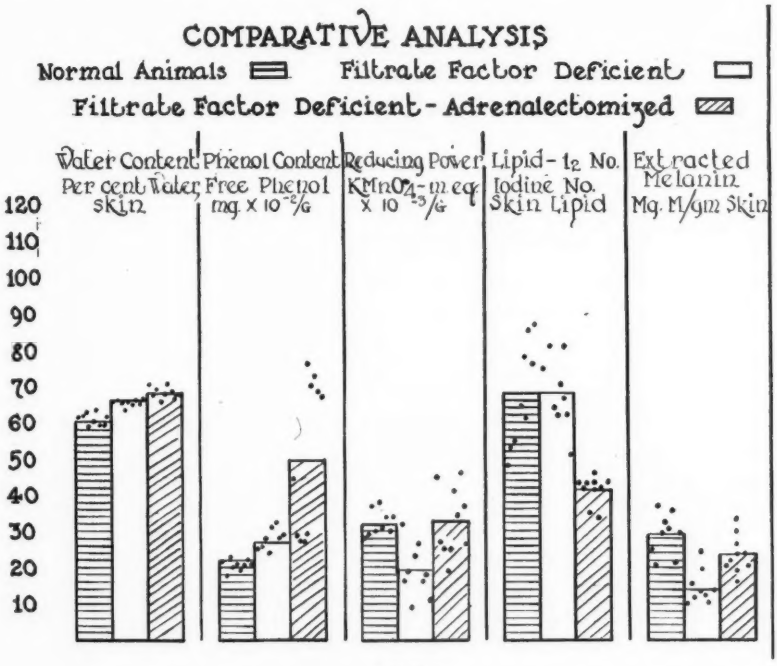

FIG. 2

chemical confirmation of the repigmentation that was observed in the animals. In the filtrate factor deficient rats much of the melanin was present in the tissue in colorless form, but was oxidized to its colored form during the extraction process.

Effects of hormone therapy. Repigmentation of experimentally produced achromotrichia is brought about by the chemical reaction promoted by adrenalectomy. We have attempted to determine the role of the adrenal gland in repigmentation by treating groups of filtrate factor deficient adrenalectomized rats with both desoxycorticosterone acetate (0.5 mg/rat/day) and with adrenal cortical extract (0.4cc/rat/day). In the doses used DCA represented 4–8 rat growth units, and the 0.4cc of adrenal cortical extract represented 1–2 rat

TABLE 4. RATS FED FILTRATE FACTOR DEFICIENT DIET AND ADRENALECTOMIZED. MAINTAINED ON 1% NaCl AND CORTIN 0.4 CC. DAILY (INJECTED)

Days on diet pre-op.	Survival days	Weight grams	% H_2O skin	Phenol Mg/Gm. tissue	Reducing power Meq. $KMnO_4$/Gm tissue	Lipid		Tyrosinase MgM/Gm tissue	Melanin MgM/Gm T.
						Gm/Gm tissue	Iodine #		
30	4	80	70.5	.640	.049	.003	54	25.6	24.6
	4	79	71.0	.706	.043	.005	42	25.6	18.6
	9	103	69.4	.589	.032	.005	46	16.8	32.0
	9	118	70.8	.547	.028	.009	33	16.0	12.4
	12	103	67.8	.626	.029	.009	31	32.0	17.4
	17	94	67.1	.372	.018	.008	24	24.0	13.0
	9.2	96.2	69.4	.580	.033	.007	38	23.5	19.7

RATS FED FILTRATE FACTOR DEFICIENT DIET AND ADRENALECTOMIZED. MAINTAINED ON 1% NaCl AND DESOXYCORTICOSTERONE ACETATE 0.5 MG. DAILY (INJECTED).

27	17	103	74.4	.159	.026	.016	47	31.1	9.3
27	20	103	78.0	.142	.016	.012	40	18.6	10.4
33	7	92	68.8	.265	.019	.034	33	25.5	11.4
33	8	86	70.1	.155	.007	.027	42	36.3	12.2
33	13	119	69.9	.136	.008	.019	35	35.1	13.5
33	14	101	69.7	.153	.006	.017	39	19.5	14.3
32	13.6	100.6	71.8	.168	.014	.021	40	27.7	11.9

growth units (Kuisenga, Nelson and Cortland, 1940). On the basis of cat units for maintenance, the dosages were more comparable as 0.4cc Upjohn Cortin equals 1–2 cat units; 1.0 mg DCA equals 1–2 cat units. (Hartman and Spoor, 1940). Neither form of therapy prolonged the survival of the deficient rats appreciably, but as the animals were already receiving maintenance oral administration of 1% NaCl this is not surprising. The individual chemical data from both groups of hormone treated animals is reported in Table 4. Table 5 gives the comparison of the average values for the groups of filtrate factor deficient rats that were adrenalectomized, hormone treated, and on the diet alone. Visually the DCA treated animals did not become pigmented to the same degree as did the adrenalectomized controls. The color of the skin of the adrenalectomized rats treated with the adrenal cortical extract appeared similar to the color of

TABLE 5. COMPARISON—TREATED AND UNTREATED ADRENALECTOMIZED RATS

Constituent	F.F.D. Adr.	F.F.D. Adr. + Doca	F.F.D. Adr. + Cortin	F.F.D.
No. of rats	10	6	6	9
Weight—gms.	94	101	96	74
Survival of exp. condition days	8.4	13.2	9.2	60 Day Def.
% H^2O skin	67.8	71.8	69.4	65.7
Phenol Mg/Gm tissue	0.498	0.168	0.580	0.275
Reducing power Meq. $KMnO_4$/Gm	0.033	0.014	0.033	0.019
Lipid				
Gm/Gm T.	0.009	0.021	0.007	0.017
Iodine #	41.2	39.8	38.3	67.9
Tyrosinase MgM/Gm tissue	28.6	27.7	23.5	33.8
Melanin MgM/Gm tissue	23.5	11.9	19.7	1.46

the skin of untreated adrenalectomized animals. The most significant finding in the DCA treated rats was a decrease in the high phenol content ordinarily found following adrenalectomy. The phenol level was below that found in unoperated rats on the filtrate factor deficient diet. In the rats treated with the adrenal cortical extract the phenol content remained as high as in untreated adrenalectomized controls. The reducing power of the tissue of DCA treated rats fell to the level of the filtrate factor deficient rats. No significant changes in iodine numbers were observed in either group, although the total lipid in DCA treated animals was higher than in either the untreated or the adrenal extract treated group. Tyrosinase activity showed little variation in the hormone treated animals. Extractable melanin again paralleled the degree of color observed grossly. The hides of the filtrate factor deficient adrenalectomized and adrenal extract treated rats yielded greater quantities of melanin than the normal, but the hides of the DCA treated animals yielded *even less melanin* than did the filtrate factor deficient unoperated rats.

Rats on normal diets. The results obtained on the filtrate factor deficient adrenalectomized rats treated with DCA, in which the hormone not only inhibited the deposition of melanin but also caused the chemical factors in the skin to return to a state very similar to that of the unoperated filtrate factor deficient rat, suggested that the changes in pigmentation were in some way associated with the disturbance in salt and water metabolism known to occur in the absence of the adrenal cortex. To study this effect NaCl was omitted following adrenalectomy in rats fed the diet *supplemented with the filtrate factors.* The animals only survived adrenalectomy 10–12 days and in this period no increase of extractable pigment was found. Chemical analyses of the hides of this group did not show a rise in phenols but there was decrease in the normal reducing power. A slight increase in the percentage of skin water as compared to the normal rat was found. The iodine number fell appreciably and was lower than in adrenalectomized filtrate factor deficient rats given NaCl. Extractable melanin was in the low range of normal. In order to investigate further the results of changes in water balance, a group of rats receiving the Nu Chow diet was deprived of water altogether and another group was dehydrated by allowing them to drink a 2% solution of NaCl ad lib. The amounts of extractable melanin in the hides of both groups approached the amounts present in filtrate factor deficient rats. The phenols were greatly increased in the rats on 2% NaCl and in the rats deprived of water. The individual chemical data from each animal in the 3 groups discussed above is given in Table 6. In Table 7, the data on the 3 groups is compared with the data obtained on normal animals given tap water, on filtrate factor deficient animals on 1% NaCl, on filtrate factor deficient adrenalectomized rats on 1% NaCl, and on adrenalectomized rats fed a diet

TABLE 6. ADRENALECTOMIZED RATS ON DIET SUPPLEMENTED WITH FILTRATE FACTORS*—NO NaCl

Age at adr.	Days survived after adr.	Weight at death grams	% H_2O skin	Phenol Mg/Gm tissue	Reducing power Meq. $KMnO_4$/Gm tissue	Lipid		Tyrosinase MgM/Gm tissue	Melanin MgM/Gm tis.
						Gm/Gm tissue	Iodine #		
65	10	110	66.3	.184	.013	.014	37.5	21.5	23.4
	10	118	64.3	.244	.024	.013	23.0	20.6	23.2
	12	126	66.2	.181	.017	.012	28.0	23.4	16.5
Aver.	10.6	118	65.6	.203	.018	.013	29.5	21.8	21.0
NORMAL RATS ON NU CHOW DIET PLUS 2% NaCl DRINKING WATER									
38	16	33	57.5	0.710	.045	.031	19.0		20.5
	17	37	—	1.030	.060	.020	23.0	No	18.7
	17	34	—	0.894	.033	.017	23.0	determi-	15.5
	16	35	59.9	0.943	.034	.027	22.0	nations	16.7
	16.5	37.2	58.7	0.894	.043	.024	21.7		17.8
NORMAL RATS ON NU CHOW DIET DEPRIVED OF DRINKING WATER									
55	14	43	59.9	.275	.031	.024	63.2	20.2	14.3
	15	42	60.0	.331	.027	.019	43.6	26.9	10.8
	15	56	60.8	.402	.017	.014	53.5	38.2	13.7
	17	54	61.7	.497	.017	.023	51.5	34.5	13.7
	18	57	59.4	.420	.022	.023	60.3	22.5	16.6
	15.8	50.4	60.4	.385	.023	.021	54.4	28.5	13.8

* A rice bran extract supplied by the Galen Co.

supplemented with the filtrate factor, but given no sodium chloride. The groups are numbered in the table as follows:—Group I: *Normal animals*—these were animals fed the Nu Chow diet and received water ad lib. Groups II and III: These animals were also fed the Nu Chow

TABLE 7. INFLUENCE OF ADRENALECTOMY; WATER LACK; HIGH SALT; FILTRATE FACTOR DEFICIENCY

Group	I Normal rats Nu Chow diet	II Normal rats on 2% NaCl	III Normal rats no water	IV Unoperated F.F.D. rats 1% NaCl	V Adrenalectomized F.F.D. rats 1% NaCl	VI Adren. rats on diet with filtrate factors No NaCl
No. of rats	9	4	5	9	10	3
Weight—gms.	104.	37.	50.	74.	94.	118.
Age at death, days	47	55	70	95	73	76
Survival of exp. condition, days	47	16.5	16	60	8.4	10.6
% H_2O skin	60.9	58.7	60.4	65.7	67.8	65.6
Phenol Mg/Gm tissue	0.206	0.894	0.385	0.275	0.498	0.203
Reducing power Meq. $KMnO_4$/Gm	0.032	0.043	0.023	0.019	0.033	0.018
Lipid Gm/Gm T.	0.031	0.024	0.021	0.017	0.009	0.013
Iodine #	67.6	21.7	54.4	67.9	41.4	29.5
Tyrosinase MgM/Gm tissue	31.8	No Determ.	28.5	33.8	28.6	21.8
Melanin MgM/Gm tissue	29.0	17.8	13.8	14.6	23.4	21.0

diet but Group II received 2% NaCl ad lib, and Group III was completely deprived of water. Group IV consisted of animals given the filtrate factor deficient diet and 1% NaCl when they were 35 days of age. Group V consisted of filtrate factor deficient animals that were adrenalectomized after having been on the deficient diet for 30 to 35 days. Group VI consisted of animals that were fed the deficient diet supplemented with a rice bran extract containing the filtrate factor at 35 days of age, and after 30 days on this diet they were adrenalectomized, continued on the diet, but given no salt. Comparison of the determinations recorded in Table 7 indicate that some of the changes induced by filtrate factor deficiency alone can occur in normal animals deprived of water (Group III). The extractable melanin was almost identical in the 2 experimental groups; e.g., 13.8 mgm/gm tissue in normals, 14.6 mgm/gm tissue in the deficient rats. The change in the reducing power was in the same direction in both groups, although the deviation from the normal was greater in the filtrate factor deficient animals. The phenols rose in both groups; the rise was greater in the animals deprived of water. These were the significant changes and indicate that in respect to the substances mentioned, water deprivation may produce changes that simulate filtrate factor deficiency.

Adrenalectomized rats on a diet supplemented with the filtrate factors but not maintained on 1% NaCl did not show the same rise in phenols or the same increase in reducing power characteristic of the 1% NaCl treated filtrate factor deficient adrenalectomized animals. The absolute extractable melanin in Group VI was less than in normal rats of the same age. One would anticipate from the rise in melanin occurring in filtrate factor deficient adrenalectomized rats that a marked increase would have occurred in rats maintained on a diet *supplemented* with the filtrate factors. Such an increase in melanin pigment was borne out pathologically in the skin sections of the adrenalectomized animals previously reported. In fact, the deposition of melanin in the hair follicles and bulbs in adrenalectomized rats fed the filtrate factors was the most striking observed in any group (Ralli and Graef, 1943). The fact that the extractable melanin in adrenalectomized rats on the complete diet, deprived of salt, was less than in normal animals of the same age suggests that the lack of NaCl interfered in some way with the deposition of melanin that follows adrenalectomy. The expected rise in phenols did not occur in Group VI and the reducing power fell. This finding is consistent with a decrease in extractable melanin. In other words, the whole picture points to the importance of salt in the deposition of melanin following adrenalectomy.

The normal animals given 2% NaCl showed an increase in phenols and in reducing power. Extractable melanin, however, was low compared to that obtained in the normal animal not on NaCl. It should

be pointed out that the data are not strictly comparable because of the great weight loss produced by the high salt intake. Normal animals dehydrated by water deprivation (Group V) showed an even greater decrease in melanin deposition with much less increase in phenols and a greater decrease in reducing power than did the normal rats dehydrated with 2% NaCl. The findings further support the suggestion that NaCl plays some part in melanin production or deposition.

SUMMARY

Chemical analyses of phenolic compounds (precursors to melanin), reducing power, tyrosinase-like activity, lipid and preformed melanin were done on the hides of normal rats; rats fed diets deficient in the filtrate factors of vitamin B; of adrenalectomized rats receiving the deficient diet, some of which were treated with either the adrenal cortical extract or desoxycorticosterone acetate after adrenalectomy. Analyses were also done on the hides of rats receiving either 1% NaCl, 2% NaCl, tap water or on animals deprived of water. The diet deficient in the filtrate factors not only produced achromotrichia but also resulted in a decrease in the extractable melanin of the skins of the rats. Dehydration by means of 2% NaCl or by water deprivation produced chemical changes in the skin simulating to a certain extent those induced by filtrate factor deficiency. Adrenalectomy in rats fed the deficient diet resulted in an increase in the extractable melanin of the skin, and this was inhibited when the rats were treated with DCA. The skins of adrenalectomized rats deprived of salt but fed diets supplemented with the filtrate factors did not contain greater amounts of melanin than the skins of normal rats. The studies show that adrenalectomy not only reverses the achromotrichia produced in black rats by diets deficient in the filtrate factors but also results in an increase in the extractable melanin of the hide. Both the adrenal cortex and sodium chloride are involved in the metabolism of melanin.

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THE ANTERIOR PITUITARY AND ITS RELATION TO THE ADRENAL CORTEX IN WATER DIURESIS[1]

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IT IS WELL known, particularly from work on diabetes insipidus, that the anterior pituitary contributes to the control of water exchange by exerting a diuretic influence. This is seen most clearly when its action is unchecked by the antagonistic secretions of the neuro-hypophysis (reviewed by Fisher, Ingram and Ranson, 1938; and Gersh, 1939). The means by which the anterior pituitary mediates this effect is not fully established, but is probably multiple in nature (Richter, 1934; Schweizer, Ehrenberg and Gaunt, 1943).

A somewhat different aspect of the problem can be observed when, instead of focussing attention on the daily water exchange, the disposal of measured doses of water given by stomach tube to fasted hypophysectomized animals is observed in short-term experiments.

Our attention was drawn to this phase of the subject in 1939 when we attempted to determine whether hypophysectomized rats differed from normal ones in their sensitivity to the antidiuretic action of vasopressin. We intended to test this by the usual method of observing effects on the rate of excretion of water (5 per cent of body weight) given by stomach tube. It was found that the test was unsuitable for such purposes, because hypophysectomized rats failed to show any clear diuretic response to water given in this way. This was surprising in view of the fact that such animals under ordinary living conditions show a mild, persistent diabetes insipidus (Schweizer, *et. al.*, 1941). Walker (1939) observed, incidental to other studies, this same inability of hypophysectomized rats to excrete water normally. Chen and Geiling (1943) reported the first detailed and analytical data on the subject. We had observed previously (Gaunt, Remington and Schweizer, 1937) a marked susceptibility to water intoxication in

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hypophysectomized rats. This, however, in view of the large doses of water used, did not necessarily imply that small doses could not be handled normally.

Our observations, on which a recent preliminary report has been made (Gaunt, 1944a), confirm and extend the findings of the above workers, and in addition, provide some elucidation of the mechanism of the deficiency.

METHODS

Tap water and other test fluids (5 cc. per 100 grams body weight) were given at body temperature by stomach tube to 150 to 200 gram male rats after a 12 hour fast. In other instances, 0.9 per cent sodium chloride solutions were injected intraperitoneally. Rats which had been hypophysectomized from one to 17 weeks were compared with intact controls. The animals were kept in small individual metabolism cages during the experiments. In all cases the animals showed the usual signs of hypophyseal deficiency, but care was taken not to use animals in which the cachexia was great enough to indicate a short life expectancy. In no case did the fast and other manipulations involved in these experiments lead to any obvious deleterious effects in the test animals. The end-point taken for each experiment was the time necessary for the excretion of 50 per cent of the fluid administered. Blood pressure was measured by the method of Duncan, Hyman and Chambers (1943) and water absorption by the method of Heller and Smirk (1932). Extra-renal water loss was measured by weighing the animals at hour intervals and determining loss of weight above that due to the output of urine and feces.

RESULTS

Excretion of water given orally (*Table 1*). Normal animals excreted 50 per cent of the water given in an average of 82 minutes—a figure in close agreement with that reported in diuresis experiments by many other observers. Hypophysectomized animals usually required from 8 to 9 hours to excrete similar quantities of fluid, although the individual variation, in the operated animals, was very great.

The rate of water excretion in hypophysectomized animals was so slow that by the time 50 per cent of the water administered had been eliminated by the kidney, the animals had lost the other 50 per cent by extra-renal routes. The extra-renal loss itself, probably because of decreased metabolism, was lower in hypophysectomized than in normal animals.

Walker found that several weeks after hypophysectomy rats showed some improvement in their ability to excrete water. We observed (Table 3) no difference in such performance at any interval up to 16 weeks after operation.

Excretion of normal saline given orally (*Table 1*). It is well known, and was confirmed here, that isotonic saline given by mouth is excreted more slowly than water by normal animals. Hypophysectomized animals, however, excreted the saline at essentially the same rate as water.

Excretion of normal saline given intraperitoneally (Table 1). Normal animals excrete 0.9 per cent sodium chloride more slowly when it is given intraperitoneally than when given by mouth. This difference is probably due to different rates of absorption from the two sites.

Hypophysectomized rats excreted saline given by mouth, saline administered intraperitoneally, and tap water given by mouth at essentially the same rates. The lack of a difference between the oral

TABLE 1. AVERAGE TIME FOR EXCRETION OF 50 PER CENT OF ADMINISTERED FLUID (5 PER CENT OF BODY WEIGHT) IN NORMAL AND HYPOPHYSECTOMIZED RATS

	Tap water—Oral			0.9% NaCl—Oral		
	No. of animals	Time for 50% excretion ±S.E. & range (min.)	Extra-renal loss (cc. per hour)	No. of animals	Time for 50% excretion ±S.E. & range (min.)	Extra-renal loss (cc. per hour)
Normal	56	82 ± 2.8 43–133	1.32 (5 cases)	72	185 ±11.5 68–399	0.97
Fasted hypophysectomized	56	530 ±42.8 111–1330	.53 (27 cases)	55	464 ±27.5 141–1070	0.48
Unfasted hypophysectomized	6	533 343–990	.44			
	0.9% NaCl—Intraperitoneal			5.5% Glucose—Oral		
Normal	45	291 ±10.4 146–271	0.79	11	79 ± 3.0 66–110	0.87
Fasted hypophysectomized	14	536 ±42.3 345–975	0.47	22	428 ±88.2 86–1400	0.60

and intraperitoneal routes is consistent with the finding, reported below, that intestinal absorption is impaired thus making it more nearly equal the absorption rate from the abdominal cavity.

Cause of delayed diuresis in hypophysectomized animals.

Blood pressure. During the course of these experiments hypophysectomized animals did not show signs of collapse or of water intoxication which might have accounted for the lack of diuresis. It is known, however, that hypophysectomized rats are exceedingly susceptible to shock-inducing stimuli. We thought it possible that the forcing of a stomach tube and other manipulations involved in the experiments, mild though they were, might provoke a shock-like fall in blood pressure and thus account for some of the results obtained. No consistent changes in pressure were found, however, within a 5-hour period of observation after water was given. Blood pressure at

the start of the experiment was lower in hypophysectomized (82 mm. Hg.) than in the control rats (91 mm. Hg.). This fact that blood pressure is chronically low in hypophysectomized rats (Leathem and Drill, 1943; and Joseph, *et al*, 1943) may interfere with the establishment of maximal diuresis, but since such animals can maintain a high daily water exchange under normal conditions, it cannot be considered an important cause of the results seen here. These findings on blood pressure are consistent with those seen in adrenalectomized animals given intoxicating doses of water (Gaunt, 1944b).

Fasting. The lack of diuresis in hypophysectomized animals is not

TABLE 2. MEAN FIGURES ON ABSORPTION AND DISPOSAL OF WATER GIVEN BY MOUTH IN NORMAL AND HYPOPHYSECTOMIZED RATS
(Animals killed 1 hour after receiving 2nd of 2 doses of water. All figures in cc. per 100 gm. body weight.)

Treatment	Water given	Water excreted	Water in stomach	Water in intestine	Water absorbed but not excreted[1]	Water unaccounted for[2]	No. of animals
Normal controls	9.7	3.7	0.07	0.8	3.9	1.2	8
Hypophysectomized 7 days	10.0	0.5	0.9	1.7	5.7	1.2	6

[1] Estimated by increase in body weight minus increase in gut weight.
[2] Represents insensible water loss, loss of weight by defecation, loss of urine by evaporation on collecting apparatus, etc.

due to the effects of the preliminary fast. Animals which had been fed, those given isotonic glucose solutions as a diuretic fluid (Table 1), and those given crystalline sucrose or glucose (to furnish carbohydrate and calories but not bulk) during the "fasting" period, responded similarly to the animals subjected to the usual fasting procedure.

Intestinal absorption of water (*Table 2*). Walker suggested that the delayed diuresis in hypophysectomized animals may have been due to delayed intestinal absorption. We attempted to determine by direct measurement if this was true. The excised stomachs and intestines were weighed a set interval after water was given and these weights compared to those of the same organs from animals similarly fasted but which had received no water.

In these experiments, two doses of water were given an hour apart and the amounts absorbed measured one hour after administration of the second dose. This gave larger and more reliable volumes of water with which to work than when the usual smaller doses were used, and also gave us a basis for direct comparison with adrenalectomized animals which respond to water similarly, and on which comparable figures were already available.

A definite delay in absorption was observed, but one probably less severe than in adrenalectomized rats. It was, moreover, a poten-

tial, rather than a real cause of the delayed diuresis. Even though the water was absorbed slowly, it accumulated in the bodies of hypophysectomized animals and could not be excreted normally.

These results—namely, a delayed absorption and inadequate

TABLE 3. AVERAGE TIME FOR EXCRETION OF 50 PER CENT OF FLUID (5 PER CENT OF BODY WEIGHT) ADMINISTERED ORALLY TO HYPOPHYSECTOMIZED RATS AT VARYING INTERVALS FOLLOWING OPERATION

	Days after hypophysectomy	No. of animals	Time for 50% excretion range (min.)
Tap water	7	11	661 149–1000
	14	11	550 221–1330
	21	16	570 141–1300
	32	8	346 111– 740
	44–110	8	535 280–900
0.9% NaCl	7	23	459 141–1070
	14	8	405 206–666
	21	8	434 270–800
	28	8	477 415–780
	35	6	490 297–715
	>40	2	650 400–900

elimination of water that was absorbed—resemble qualitatively those seen in adrenalectomized rats.

Replacement therapy (Table 4).

Anterior pituitary extract. The rate of water diuresis was not increased in hypophysectomized animals in which most other deficiency symptoms were relieved by treatment for 3 or more weeks with a crude beef anterior pituitary extract. The preparation and properties of this extract, including its effect in restoring diabetes insipidus, have been described previously (Schweizer, *et al.*, 1941; Dolin, Joseph and Gaunt, 1941). With the doses of extract used (equivalent of 0.16 gm. fresh tissue daily) body weight increased at a rate greater than

TABLE 4. AVERAGE TIME FOR EXCRETION OF 50 PER CENT OF FLUID ADMINISTERED (5 PER CENT OF BODY WEIGHT) IN HYPOPHYSECTOMIZED RATS RECEIVING VARIOUS TREATMENTS

	No. of animals	Time for 50% excretion range (Min.)	Extra-renal loss (cc. per Hour)	Treatment
Tap water oral	56	530 111–1330	0.53 (27 cases)	None
	15	578 212–1638	0.50 (9 cases)	APE[1]—chronic
	4	517 245–750	—	APE[1]+Thyroid[2]—chronic
	8	600 104–900	0.59	Thyroid[2]—chronic
	4	213 131–350	0.49	Cortin[3]—chronic
	8	215 91–293	0.57	Cortin[4]—Acute
	7	131 91–150	—	Cortin[5]—Acute
	15	156 72–203	0.71 (12 cases)	DCA[6]—Acute
	13	398 154–660	0.66 (5 cases)	DCA[7]—Acute
0.9% NaCl oral	6	315 217–397	0.70	DCA[6]—Acute
0.9% NaCl intraperitoneal	5	349 202–462	0.43	DCA[6]—Acute

[1] 2–10 weeks.
[2] 1–3 weeks.
[3] 0.5 cc. Eschatin daily for 17 days before experiment; hypophysectomized 2–3 months.
[4] 1.5 cc. Eschatin, subcutaneous.
[5] 2.5 cc. Eschatin, subcutaneous.
[6] 2 mg. Desoxycorticosterone acetate.
[7] 0.5 mg. Desoxycorticosterone acetate.

normal, appetite was increased to above normal levels and the animals appeared in excellent health. No injections were made 24 hours before diuresis tests, because of possible complications from contaminating antidiuretic substances in the extracts. Under these conditions, there was no suggestion of improvement in the diuretic response to water. It is significant in view of the findings reported below, that while these extracts restore thyroid function in part, and hence oxygen consumption, they have little if any effect on the atrophic adrenal cortices.

Thyroid. Thyroid atrophy and hypofunction is one result of hypophysectomy. Many workers (e.g. Pick, 1929) have reported that the

thyroid is concerned in water metabolism. Desiccated thyroid was fed in doses that restored the oxygen consumption of the hypophysectomized rats to normal or above (specific figures quoted by Schweizer, *et al.*, 1943). In some cases this treatment was combined with anterior pituitary extract as described above. In neither instance was the diuretic response to water improved, and the apparent further inhibition of diuresis seen in the table is probably of no significance.

Adrenal cortex. It is also well known that adrenal cortical atrophy and hypofunction follow hypophysectomy. Replacement of this deficiency by either cortical extract (2.5 cc.) or desoxycorticosterone acetate (2 mg.) restored water diuresis almost to normal. Smaller doses were effective to a lesser extent. The injections of the larger doses of extract were divided as follows: 1 cc. was injected one-half hour before water was given, 1 cc. at the time water was given, and one-half cc. 30 minutes later. The water dose was reduced by the amount of fluid given in the last two injections.

The 2 mg. doses of desoxycorticosterone were given in variously divided fractions beginning at 18 hours and ending 1 hour before diuresis tests.

In addition to the effects on water given by mouth, desoxycorticosterone acetate accelerated the rate at which normal saline was excreted when the latter was given either intraperitoneally or by mouth.

While the reparative action of cortical substances was unmistakable, it is noteworthy that in no series, with the dosage used, was diuresis restored completely to normal. Larger doses might have produced such normal responses, but we consider it more probable that the deficiency was not entirely due to cortical hypofunction and hence not entirely relieved by therapy with cortical hormones.

DISCUSSION

It would appear that the lack of normal water diuresis in hypophysectomized rats was primarily, but probably not entirely, due to the atrophic and hypofunctional state of the adrenal cortices of these animals. This suggests again the probability that water diuresis is invariably deficient in the absence of normal adrenal cortical function, and supports the use of water diuresis tests in the diagnosis of adrenal insufficiency (Robinson, Power and Kepler, 1941; Gaunt 1944a).

The primary responsibility of the adrenal is indicated by the effects of cortical replacement therapy, and by the apparent identity of deficiency symptoms seen in adrenalectomized and hypophysectomized animals: namely, decreased absorption and inability to excrete fluid that is absorbed. The latter is perhaps related to the renal function changes known to occur in hypophysectomy (Heinbecker, Rolf and White, 1933), and is of such magnitude that the delay in absorp-

tion is relatively of little importance as a cause of the retarded diuresis.

This does not mean necessarily that the entire action of the anterior pituitary on water metabolism is mediated through the adrenal cortex. Pituitary extracts of the type found ineffective here will in fact help restore diabetes insipidus in hypophysectomized animals. But under the conditions of these experiments, none of the diuretic hormonal agents appears to be able to act in the absence of cortical hormones. The same has been found to be true in the case of hyperthyroid animals which show a greatly enhanced water diuresis only in the presence of functional adrenals (Gaunt, Cordsen and Liling, 1944).

It has been shown by Chen and Geiling that the loss of a normal diuretic response to water following hypophysectomy is due entirely to removal of the anterior pituitary. The presence or absence of the posterior lobe did not modify their results.

SUMMARY

Previous findings that hypophysectomized rats show a greatly delayed diuretic response to water were confirmed. This effect of hypophysectomy is persistent and unchanging between 1 and 17 weeks after operation. It is not due to hypotension or to fasting.

Hypophysectomized rats, in contrast to normal ones, excrete normal saline solutions, given either orally or intraperitoneally, at about the same rate as they do water given by mouth.

There is a delay in absorption of water given orally, but the predominant cause of the delayed diuresis lies in an inability to excrete normally water that is absorbed.

The deficiency is not repaired by an anterior pituitary extract which restores growth and appetite to normal or above, nor by doses of thyroxin which restore basal metabolism to normal, nor by a combination of the two. It is, however, almost completely repaired by relatively small doses of cortical extract or desoxycorticosterone acetate, and for this and other reasons is considered to be primarily due to adrenal cortical deficiency. These observations provide another instance supporting the use of water diuresis tests (Robinson-Power-Kepler) in the diagnosis of mild adrenal insufficiency.

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IMMUNE REACTIONS IN RABBITS AND RATS AFTER INJECTIONS OF THYROID GLOBULIN

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THE SPECIFIC response elicited in an animal by parenteral administration of hormones of protein character tends to decrease with prolonged treatment and eventually to end in complete refractoriness manifested by signs of deficiency if the treatment is discontinued. This observation admits of various interpretations which do not necessarily contradict one another because various factors may be at play simultaneously, and also because the different theories applied are not sharply enough defined as to exclude the possibility that the difference exists more in names than in facts or conceptions of them. Four explanations may be proposed: 1. Development of tolerance similar to that occurring by the use of morphine or arsenic. 2. An endocrine mechanism: The injected hormone suppresses the gland active in elaborating the same, either directly or by way of the pituitary. This effect, in case of thyroid, is also obtained by oral administration, as has been shown by Farquharson (1941) and Reinhoff (1940, 1941). 3. Antihormones are formed, i.e. substances directly antagonistic to the hormonic component of the injected material and, therefore, also largely specific for the same endocrine unit from other species. 4. Formation of true antibodies, effective essentially against the total protein molecule and only by a spread of action effective in a limited way against the corresponding hormones of different species.

In evaluating the results obtained by Lerman (1942) in the immunization of rabbits with thyroid globulin and those by others discussed in an editorial in the Journal of Clinical Endocrinology (1942), one gains the impression that probably both an endocrine mechanism and immunization are at work. Lerman was unable to duplicate in man the process of immunization as observed in the rabbit, which he explained by the fact that man is not a good antibody producer. This suggested the present investigation, a comparison of the effect of

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thyroid globulin in the rabbit and in the rat, the latter being recognized as a poor producer of antibodies.

METHODS

The thyroid globulin used in these experiments was prepared by extracting human thyroids obtained by surgery, or pigs' thyroids, with 1% NaCl solution at a pH of 8.0. The extract was precipitated by dialysis against the same volume of saturated ammonium sulfate solution buffered at, and later adjusted to, a pH of 4.5. The precipitate was washed with ½ saturated ammonium sulfate solution, suspended in water, and the mixture was dialized against buffer solution of pH 7.8–8.0. The insoluble part was centrifuged off and the same purifying process repeated. The final solution was dialyzed

TABLE 1. PRECIPITIN—TITRATION OF SERUM

	Dilution									
	Human thyroidglobulin					Pigs' thyroidglobulin				
	10	100	1,000	5,000	10,000	10	100	1,000	5,000	10,000
I	++	+	+	±	−					
II	+++	++	++	+	±		+	−		
III						+++	++	++	+	±
IV						++	+	±	±	−
V						+++	++	++	+	−

Thyroids of all rabbits in resting stage.
Pituitaries slightly reduced in size.
Rabbit I & II—Human thyroidglobulin
8 weeks treatment 0.13 mg. I per dose.
III, IV & V—Pigs' thyroidglobulin
8 weeks treatment 0.2 mg. I per dose.

against 0.5% NaCl solution until free from ammonium sulfate and filtered through Chamberland filters.

The rabbits used for immunization had an average weight of 2.5 kg. and were kept on a diet of Rockland Rabbit Ration supplemented daily with carrots and green vegetables. Following Lerman's procedure, they were injected alternately two and three times weekly with 2½–3 cc. of the thyroid globulin solution. There is no objection to giving the first three injections intravenously, but on repetition animals may be lost by anaphylactic shock, which does not happen if the intraperitoneal route is used. The rats used were of Sherman strain kept on a diet of three parts whole wheat and one part of dried whole milk, supplemented with lettuce. Rats were injected intraperitoneally on three consecutive days per week and changes in metabolism were determined on the fifth day.

In both groups the animals were bled at the end of treatment and the precipitin content of the serum was determined by titration with the same thyroid globulin that had been used for treatment. Furthermore, the value for passive immunization of untreated animals was tested. Normal rabbits were given the serum subcutaneously alone at first and later, preceding intraperitoneal injections of the antigenic thyroid globulin, both treatments being followed by determinations of the effect on metabolic rate. The neutralizing

effect of the rats' serum on the antigen was tested in untreated thyroidectomized rats because they respond to extremely small doses of thyroid globulin with marked increase in metabolic rate (Meyer and Wertz 1939) and thus any effect of the serum should be more noticeable.

RESULTS AND DISCUSSION

The first series of injections in rabbits yielded sera of similar potency, measured by the precipitin test, as obtained by Lerman (1942).

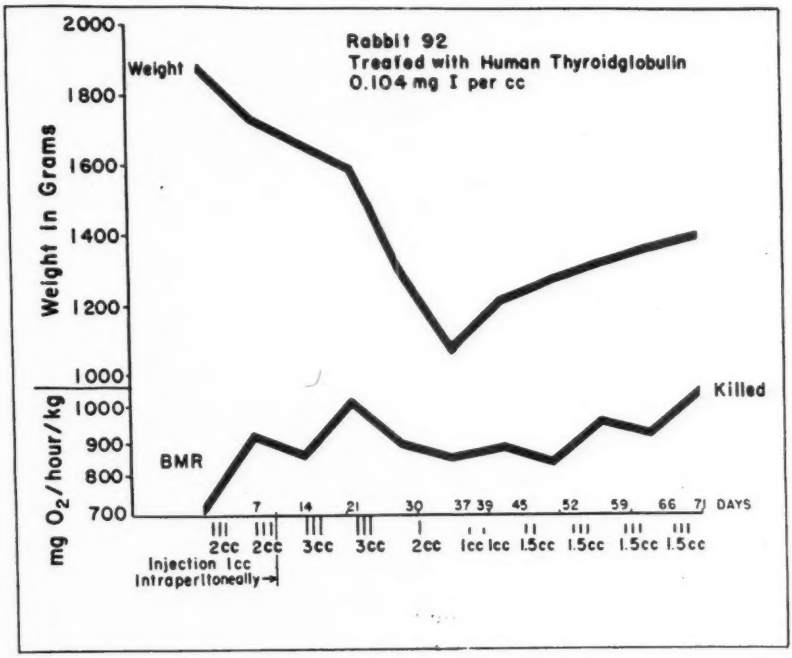

FIG. 1

Table 1 shows these results. The pigs' thyroid globulin solution used contained 1.2% globulin of 0.54% I content, supplying 0.065 mg. I per cc. The human thyroid globulin solution contained 2% of a globulin of 0.26% I content, supplying 0.041 mg. I per cc. The thyroids of all these animals were, as was expected, small, the acini lined with flat epithelium and well filled with colloid.

The appearance of the other glands may be of interest. Only the testes showed depression, with absence of spermatogenesis. The exact age of these animals was not known and it is not certain whether they were mature at the beginning of the treatment, although at the end they certainly were over six months and should have reached matu-

rity. It remained uncertain whether the effect of the injections was actually an interference with the function of the gonad or a delaying of maturation. Therefore, another male rabbit of proved fertility was treated intensively with thyroprotein for six weeks. The testes of this animal showed depression. Spermatogenesis was present but the number of spermatocytes and spermatids was reduced. Considering the enormous loss in weight (from 3.6 to 1.76 kg) during the treatment, it appears possible that the depression of spermatogenesis de-

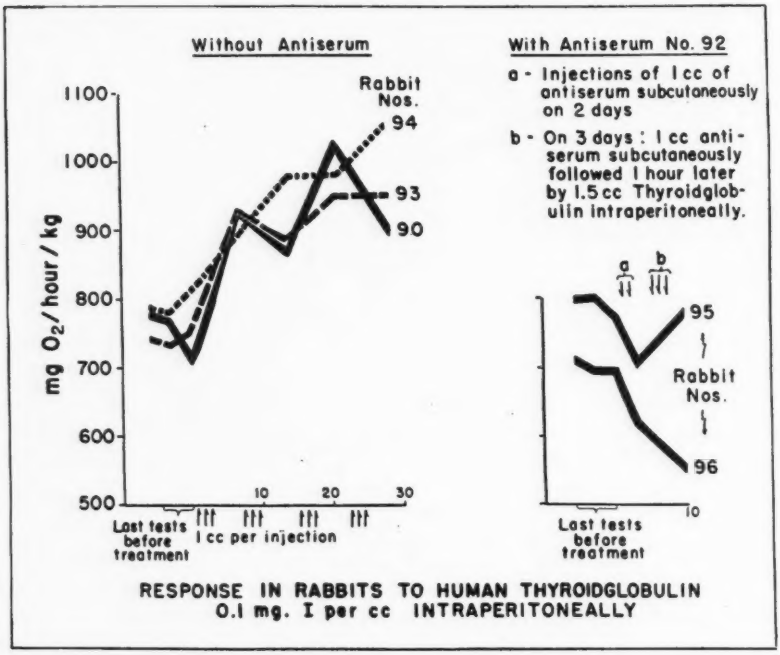

FIG. 2

pends more on the general nutritional condition than on the thyropituitary inter relationship. The pituitary weight was 22.7 mg, which may be considered as low normal.

The total response of one of the rabbits to continued injections of human thyroid globulin is shown in Fig. 1. It was found in this and other experiments that large dosages of 0.2–0.3 mg globulin-iodine lead to excessive weight loss and emaciation, that often cannot be redeemed even by discontinuation of treatment. We lost four animals by using the dose mentioned and not reducing it early enough when the weight dropped at a rapid rate.

The curves presented show how high dosage caused an excessive

loss in weight, which continued while the metabolic rate dropped in response to drastic reduction in treatment. Later, there was a gain in weight although the metabolic rate was again ascending. The thyroid of this animal was in inactive state as described. However, small as it was, it still contained active thyroid hormone. The two lobes were fed on two days to a thyroidectomized rat and caused an increase in

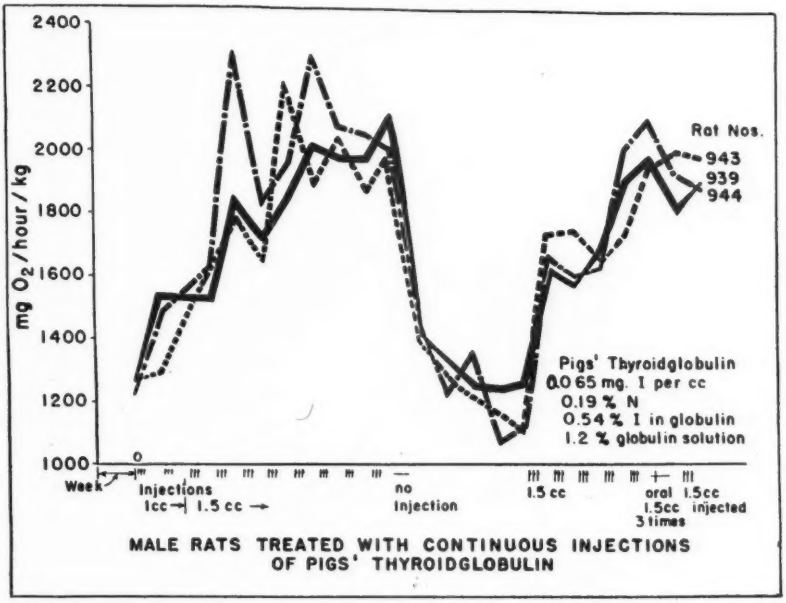

FIG. 3

metabolic rate from 985 mg O_2/hr./kg to 1305 on the fourth day, an increase of 32%.

The serum obtained from this rabbit (92) had a precipitin titer of 1:1,000. That there were actual antibodies formed was proven by injecting the serum into normal rabbits with and without administration of thyroid globulin-antigen.

Figure 2 shows first the positive response in B.M.R. elicited in three rabbits by three injections of 1 cc. thyroid globulin. The second two curves represent two other rabbits. Two subcutaneous injections of 1 cc. antiserum #92 caused a drop in metabolic rate to subnormal levels. The rabbits subsequently received on each of three successive days 1 cc. antiserum #92 subcutaneously and one hour later 1.5 cc. thyroid globulin intraperitoneally. Rabbit 95 responded with an increase in metabolism to a normal level suggesting that the two types

of injections had practically neutralized each other. The second rabbit, however, underwent a further drop in metabolic rate. There seemed to be an excess of antiserum present, capable of neutralizing not only the injected hormonic material but in addition, totally or partially, the animal's own thyroid secretion.

The reaction of rats to treatment differed in various respects from that of the rabbits.

Figure 3 shows the responses of three rats to a series of pigs' thyroid globulin injections. On discontinuation of injections the meta-

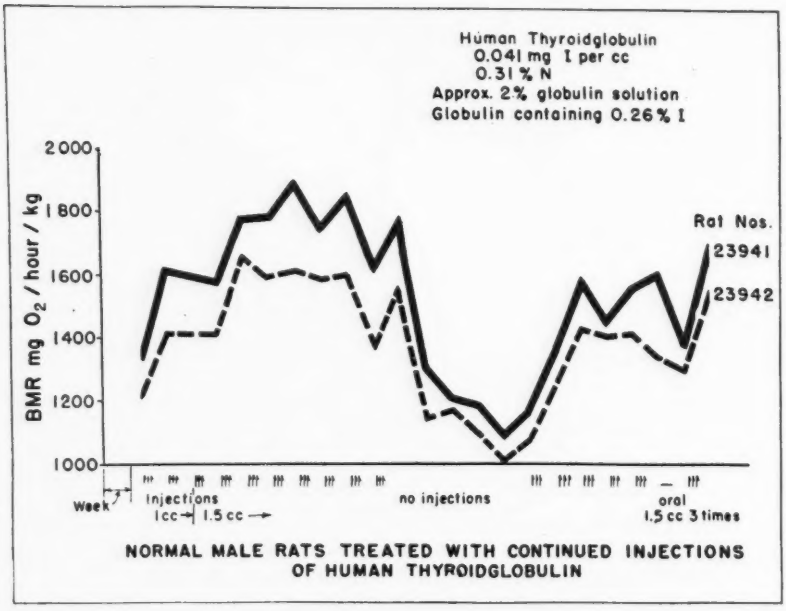

FIG. 4

bolic rate dropped to slightly below normal, proving a hypofunction of the animals' own thyroid. Subsequent injections brought the metabolism approximately up as high as before. There was no change when the medication was given by mouth instead of by intraperitoneal route.

Figure 4 presents the corresponding curves obtained with human thyroid globulin, which are similar but lower due to the fact that the human globulin contained only two-thirds as much iodine as the pigs' material. These rats showed, on autopsy, a high grade depression of the thyroid but unimpaired spermatogenesis. The serum gave a precipitin titer of 1:10 or less, showing a complete absence of this type of antibody reaction.

The sera were tested on thyroidectomized rats by injecting aliquot parts subcutaneously one and two hours preceding the antigenic thyroid globulin. The results, together with tests using the thyroid globulin without serum, are presented in table 2. Since the doses of thyroid globulin amounted only to 0.01–0.02 cc. and the total serum of one immunized rat was injected against it, the failure to prevent an increase in metabolic rate proved definitely the absence of any immunity.

Conclusions: The present investigation confirms again the known fact that the rabbit is a good producer of antibodies to injected anti-

TABLE 2. THYROIDECTOMIZED RATS INJECTED ON 3 CONSECUTIVE DAYS WITH THYROIDGLOBULIN. 0.6 MICROGRAM I PER 10 GRAMS-WEIGHT WITHOUT OR PRECEDED BY CORRESPONDING ANTI-SERUM.

Rat No.	Thyroid-globulin	Anti-serum from rat	Metabolism mg. O_2 per hr. per kg.		% Increase
			Before treatment	After treatment	
929	human	—	945	1205	28
957	human	—	950	1360	43
116	human	anti-human	955	1195	24
152	human	anti-human	980	1150	17
199	pigs	—	1020	1235	23
202	pigs	—	970	1190	22
248	pigs	—	980	1240	26
157	pigs	anti-pig	1060	1430	35
293	pigs	anti-pig	960	1195	21

gens, in this case thyroid globulin, in contrast to the rat where it was not possible to detect any immunity reaction. The immune serum formed in the rabbit was effective in producing passive immunization in normal rabbits. It inhibited the hormonic secretion of the animals' own thyroid as evidenced by a reduction in metabolic rate, and also prevented the increase in metabolic rate in response to injections of the same thyroid globulin that had been used in the process of immunization.

The thyroids of both species reacted to the thyroid globulin injections in a similar way. The gland showed signs of hypoactivity, namely considerable reduction in size, flat acinar epithelium and ample colloid within the round acini. These changes in the thyroid have no relationship to the formation of antibodies, but must be considered as a consequence of the depressing effect of increased quantities of thyroid hormone in the circulation, either on the thyroid. directly or by way of the pituitary.

Further investigations are intended to show: 1. The effect of prolonged treatment with anti-thyroid serum on the animals' thyroid. 2. The possibility of passive immunization of the rat with rabbit serum.

SUMMARY

Intraperitoneal injections of heterogenous thyroid globulin caused in rabbits a depression of the animal's thyroid, in size as well as in structure, and the formation of antibodies giving a percipitin test at dilutions up to 1:10,000. The serum of such rabbits, injected into untreated normal rabbits, produced a drop in metabolic rate and was able to prevent, and even overcompensate for, the metabolic effect of subsequently injected thyroid globulin of the type used for immunizations. In rats the depressor effect on thyroid was obtained also, but the serum contained no antibodies as shown by a negative precipitin test and complete failure to inhibit the effect of thyroid globulin injections in thyroidectomized rats.

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RELATION BETWEEN LIGHT-DARK RHYTHMS AND HOUR OF LAY OF EGGS EXPERIMENTALLY RETAINED IN THE HEN

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IN A PREVIOUS report (Rothchild and Fraps, 1944) we showed that the time of normal lay of the hen's egg appeared to be under the immediate control of the ruptured follicle from which the egg originated. The salient points in this study were as follows: lay of the egg occurred at the normal time in the presence of the ruptured follicle whether the next maturing follicle was excised or not, but was usually delayed for one or more days in the absence of the ruptured follicle. Some evidence also indicated that the maturing follicle, at or about the time of its ovulation, participated in this control. Several hens which laid their eggs at the expected normal time, but in the absence of the ruptured follicle, did so in close association with ovulation of a maturing follicle, and of the 31 birds reported on in this study which held their eggs following removal of the ruptured follicle, 14 laid their eggs, after varying periods of delay, concomitantly with the first ovulation which occurred following operation.

However, the remaining 17 birds laid the retained egg well before the occurrence of ovulation. This observation suggested that some other agency (probably outside the ovary) was involved in lay of retained eggs, and possibly also in the normal processes of oviposition. The limited data available from these birds suggested, but did not prove, that lay of these retained eggs occurred in association with the lighted hours of the normal regimen (14 hours of light, 10 hours of dark). We therefore decided to re-examine this indicated relationship between light-dark sequence and the occurrence of lay in the absence of the known ovarian controlling agents. The results of our tests, reported here, leave little doubt that the activity of the extra-ovarian factor involved in lay of held eggs is associated with lighted periods of light dark sequencies.

Ovulation and lay in the normal hen. Regular layers, in good production, lay an egg a day over a number of successive days, miss lay on a single day, then approximately repeat the cycle. The eggs laid on successive days constitute a clutch, and the interval from ovulation to lay of any egg in such a clutch can be predicted within rather narrow limits (± 1 hour) from the in-

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dividual hen's record of lay. Under conditions of lighting maintained in this laboratory (light 6:00 A.M. to 8:00 P.M.) during the time these experiments were run, the first follicle of a clutch is ovulated during the early morning hours (4:00 A.M. to 8:00 A.M.) and laid about 26–27 hours later. The next follicle of the same clutch is ovulated about 30 minutes after the preceding lay, and this close correlation between hour of lay and a succeeding ovulation is maintained for all follicles in a given clutch (Phillips and Warren, 1937). The interval from ovulation to lay, however, is not constant, but is somewhat longer at the beginning of the clutch, reaches a minimum in the middle of the clutch, and lengthens again as the last eggs of the clutch are laid, notably so for the terminal egg of the series (Atwood, 1929, Heywang 1938).

The terminal egg of the clutch is usually laid in the afternoon hours, the exact hour depending upon the number of eggs preceding it in the series. Thus for clutches of 2 eggs, it is usually laid between noon and 2:00 P.M., while for long clutches it is laid between 3:00 to 5:00 P.M., but lay may occur as late as 8:00 P.M. In any case, the succeeding follicle fails to ovulate within the usual 30 minutes following lay of this egg, but instead does so on the next morning, some 12–20 hours later, thus initiating a new clutch (Warren and Scott, 1935).

The longer interval from ovulation to lay of the last egg of a clutch sequence is apparently associated with the absence of ovulation. The terminal egg is therefore, as far as ovarian factors are concerned, entirely dependent upon its ruptured follicle for lay (Rothchild and Fraps, 1944).

MATERIAL AND METHODS

For these experiments we required a bird which lacked the ruptured follicle, and which would not ovulate within a period of time expected to cover the maximum length of retention of the oviducal egg. These minimum requirements were satisfied by removing at one operation the ruptured follicle from which the oviducal egg took its origin, and all the preovulatory follicles in the phase of rapid growth. The possibility of an ensuing ovulation taking place within at least a week following operation was thus excluded.

The selection of hens, their maintenance, and general operative procedure followed our previous account (Rothchild and Fraps, 1944). All birds were maintained in individual cages in batteries, and hourly collections of eggs from 8:00 A.M. to 4:00 P.M., with an additional collection at 8:00 P.M., were made daily. The birds used in this study were regular layers which missed no more than one day between clutches. The time of expected lay of the egg under study was predicted to within $\pm$one hour from the position of the egg in its clutch and the previous lay record of the individual hen.

Before operation, the presence of an oviducal egg was predicted from the lay record, checked by cloacal palpation, and confirmed by direct observation at operation. The subsequent account of normal or delayed lay refers always to this egg. A single daily cloacal palpation was performed after operation to confirm the continued presence of the egg in the bird.

Following operation all hens were observed at hourly intervals during the lighted periods and every 2 to 3 hours during periods of darkness. An additional observation was made a few minutes before the lights changed so that no question might arise regarding the time of lay of eggs near the end of either the light or dark periods.

Forty-eight birds were operated on for removal of the ruptured follicle and all preovulatory follicles in the rapid growth phase, and divided into the following two groups:

Group 1, consisting of 21 hens. The hens were returned to their cages following operation, and maintained under the standard light-dark sequence of 14 hours of light (6:00 A.M.–8:00 P.M.) and 10 hours of darkness (8:00 P.M.–6:00 A.M.) (Figure 1). The interval from ovulation of the oviducal egg

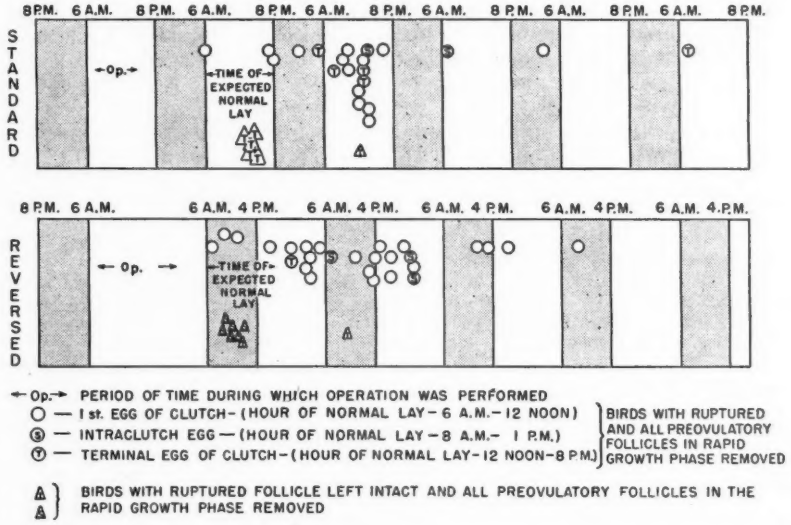
8 P.M. 6 A.M. 8 P.M. 6 A.M. 8 P.M. 6 A.M. 8 P.M. 6 A.M. 8 P.M. 6 A.M. 8 P.M. 6 A.M. 8 P.M.
STANDARD
←Op.→
←TIME OF→ EXPECTED NORMAL LAY
8 P.M. 6 A.M. 6 A.M. 4 P.M. 6 A.M. 4 P.M. 6 A.M. 4 P.M. 6 A.M. 4 P.M. 6 A.M. 4 P.M.
REVERSED
← Op. →
←TIME OF→ EXPECTED NORMAL LAY
←Op.→ PERIOD OF TIME DURING WHICH OPERATION WAS PERFORMED
○ — 1st. EGG OF CLUTCH – (HOUR OF NORMAL LAY – 6 A.M. – 12 NOON)
Ⓢ — INTRACLUTCH EGG — (HOUR OF NORMAL LAY – 8 A.M. – 1 P.M.)
Ⓣ — TERMINAL EGG OF CLUTCH – (HOUR OF NORMAL LAY – 12 NOON – 8 P.M.)
BIRDS WITH RUPTURED AND ALL PREOVULATORY FOLLICLES IN RAPID GROWTH PHASE REMOVED
BIRDS WITH RUPTURED FOLLICLE LEFT INTACT AND ALL PREOVULATORY FOLLICLES IN THE RAPID GROWTH PHASE REMOVED

FIG. 1. The relationship between light-dark sequence and the lay of eggs in the absence of the ruptured follicle and/or all preovulatory follicles in the rapid growth phase. In the upper figure is shown the alternations of light and darkness under standard conditions, in the lower figure, under reversed lighting conditions. Each shaded area represents 10 hours of darkness, each unshaded area, with the exception of the first one in the lower figure, 14 hours of light. Each circle represents lay by a bird with the ruptured follicle, and all preovulatory follicles in the rapid growth phase removed, and each triangle, lay by a bird with only the preovulatory follicles removed, except for the triangle shown in the third light period in the upper figure. The bird represented by this triangle was killed at this time, before lay had occurred. Not shown are 5 additional control birds, placed under reversed lighting, all of which laid at the expected normal hour.

to operation varied from 1.0 to 5.5 hours in 15 hens, from 7.5 to 12 hours in 4 hens, and was 25 and 27 hours in the remaining two.

Group 2, consisting of 27 hens. The birds were placed in a light-proof room following operation and maintained under lights from 4:00 P.M. to 6:00 A.M. and in darkness from 6:00 A.M. to 4:00 P.M. This lighting schedule, which is practically the reverse of the schedule under which the hens of Group 1 were maintained, was continued until all hens had laid their eggs. The time of operation and placement of hens under the altered light-dark sequence was so arranged that the first onset of darkness to follow ovulation of the oviducal egg occurred 10 hours later than under the standard conditions

(Figure 1). The time from ovulation of the oviducal egg to operation was 3 to 6 hours in 11 birds, 7 to 12 hours in 6, and 14 to 23 hours in the remaining 10.

Controls. As a control on lay of the oviducal egg by the hens in the above two groups, an exactly similar operation was performed on 19 birds, with the exception that the ruptured follicle was left intact. Six of these were returned, following operation, to the standard lighting schedule, and 13 were placed under the same reversed light-dark schedule as were the birds of Group 2. The intervals between ovulation and operation varied from 1.5 to 4.0 hours in the group of birds returned to the standard schedule, and from 1.5 to 13 hours in the group placed under the reversed light-dark schedule.

RESULTS

Of the 48 birds in Groups 1 and 2, only 4 laid at the expected normal time; the remaining 44 showed delays in lay of from 9 hours

TABLE 1. RELATION BETWEEN LIGHT AND DARKNESS AND LAY OF EGGS BY HENS LACKING BOTH THE RUPTURED FOLLICLE AND ALL PREOVULATORY FOLLICLES IN THE RAPID GROWTH PHASE, OR LACKING ONLY THE PREOVULATORY FOLLICLES

Follicles removed	Light schedule	Birds operated on	Birds holding eggs	Lay of eggs			
				Delayed		Normal	
				Laid in light	Laid in dark	Laid in light	Laid in dark
		No.	No.	No.	No.	No.	No.
Ruptured and Preovulatory*	Standard	21	20	17	3	1	0
	Reversed	27	24	17	7	0	3
Preovulatory* only	Standard	6	1†	0	0	5	0
	Reversed	13	1	0	1	0	12

* All preovulatory follicles in the phase of rapid growth.
† This bird was killed before lay had occurred, approximately 24 hours after the expected time of lay.
Standard lights: on from 6:00 A.M. to 8:00 P.M., off from 8:00 P.M. to 6:00 A.M.
Reversed lights: on from 4:00 P.M. to 6:00 A.M., off from 6:00 A.M. to 4:00 P.M.

to 4 days. Of the 19 birds which constituted the controls (ruptured follicle intact) all except 2 laid at or very close to the expected time of lay (Table 1). One bird in the group on standard light and one in the group on reversed light showed delays in lay of at least 24 hours. We can offer no explanation at this time for the cases of failure of delay in the experimental groups, and of failure of normal lay in the control group.

Figure 1 is a graphic representation of the complete experiments, and is self-explanatory. It should be pointed out however, that the time of operation as shown here does not necessarily apply to all the birds in this study, since some were operated on only a few hours before "time of expected lay." What is shown by "time of operation" is strictly the range of hours in which it was performed, and the maximum interval between it and the time of expected lay.

Group 1. Lay behavior by birds on standard light following excision

of the ruptured follicle and all preovulatory follicles in the rapid growth phase. The characteristic delay in lay appeared in 20 of the 21 birds. The length of retention of the egg beyond the expected time of lay varied from 11 hours to 4 days, but the majority of the eggs (17) were laid on or very close to the first day following that on which they were normally expected. All eggs except 3 were laid during lighted hours. The greatest single group (8) were laid between 1:00 P.M. and 4:00 P.M. or 7 to 10 hours after the onset of light, but some were laid at almost all the other lighted hours (Figure 2). No correlation could be made out between the length of retention and lay during dark hours, since the 3 birds which did so, held their eggs for 14, 16 and 67 hours, respectively beyond the hour of expected lay.

Group 2. Lay behavior by birds on reversed light following excision of the ruptured follicle and all preovulatory follicles in the rapid growth phase. The characteristic delay in lay appeared in 24 of the 27 birds. The length of retention varied from 9 hours to 3 days, but the majority (20) were laid between 9 and 36 hours after the expected time of normal lay (Figure 1).

All but 7 birds laid the held egg during the lighted portion of the day, and as in the group on standard light, the largest single number (8) were laid at an interval of 7–10 hours after the onset of light, which in this case is thus 11:00 P.M. to 2:00 A.M. The distribution of eggs according to hour laid is presented in Figure 2 and compared with that of the birds on standard light. It can be seen that most eggs are laid in approximately the same pattern, in relation to light and darkness, by both groups.

Although the length of retention in this group is on the average about the same as in the group on standard light, the strong tendency shown in the latter for lay to occur on the first day following that of expected lay was so modified that under reversed light most of the eggs were laid on either side of this period, which was a dark one. This is shown most clearly by a comparison of the frequencies of various periods of retention in the two groups. Under standard light 10 out of 20 birds held the eggs for from 23 to 30 hours beyond the time of expected lay, while under reversed light, only 2 out of 24 held for this length of time. Under reversed light 8 out of 24 birds held for 10 to 18 hours beyond the expected time of lay, compared to 5 out of 20 under standard light, and 10 out of 24 held for 30 to 36 hours, compared with 2 out of 20 birds under standard light (Figure 1).

We wish to point out the comparatively short length of time which may be involved in the appearance of the effect of light on lay. After alteration of the light-dark sequence, this effect was manifested, in at least some birds, within a period of time equivalent to a single light-dark period. It is interesting to note that a rapid response of lay to alterations of lighting conditions has been observed in the intact ring-dove by Schooley and Riddle (1944).

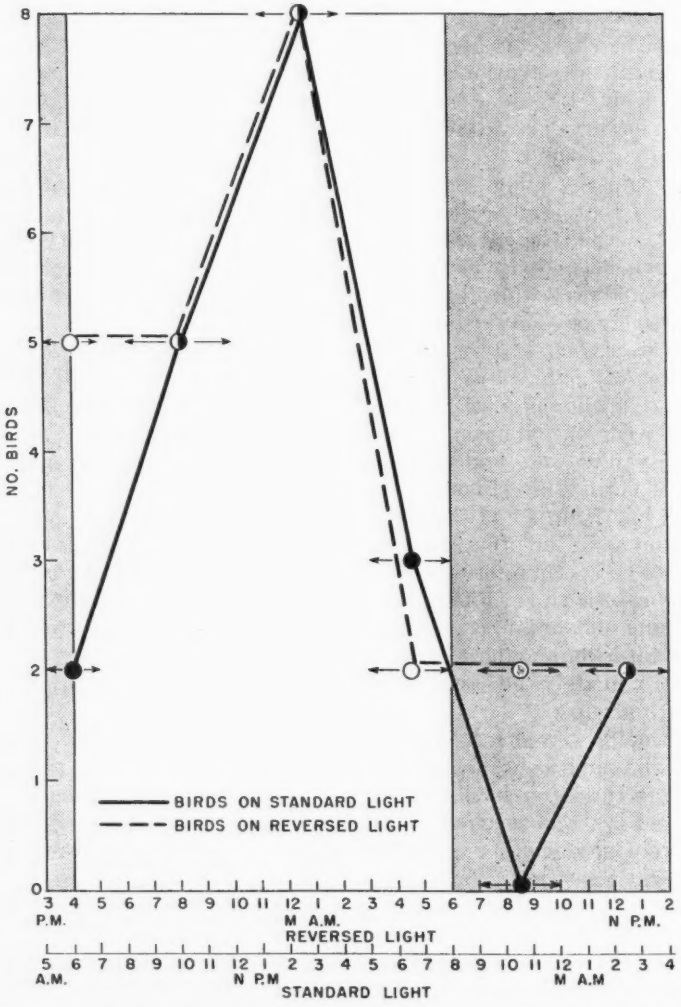
NO. BIRDS
0
1
2
3
4
5
6
7
8
BIRDS ON STANDARD LIGHT
BIRDS ON REVERSED LIGHT
3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2
P.M. M A.M. N P.M.
REVERSED LIGHT
5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4
A.M. N P.M M A.M
STANDARD LIGHT

FIG. 2. Lay of retained eggs by birds on standard and reversed light in relation to time of day. Only the hour of lay and the number of birds laying at the various hours are shown, irrespective of the length of retention. The shaded areas represent the dark portions of the day, the unshaded areas the light portions. The birds on standard light are represented by filled in circles and unbroken line, those on reversed light by the open circles and broken line. Where the values for both groups are the same a half filled in circle is used.

Controls. Lay of eggs in the presence of the ruptured follicle, and in the absence of ovulation, by birds maintained on standard and reversed light. Seventeen of the 19 birds in this group laid their eggs on the day of expected lay, within the maximum normal interval from ovulation

and irrespective of the lighting conditions at or before the time of lay.

Although lay occurred within the interval expected for normal birds, probable short delays (1 to 5 hours) appeared in 6 of the 13 birds on reversed light. That is, the time of lay was a few hours later than was expected for the particular type of egg concerned, which in these 6 cases were intraclutch eggs, but the total interval in each of these cases did not exceed the maximum normal limits for the terminal egg of clutches similar to those to which they belonged. This is a demonstration of the same phenomenon which we have noted before (Rothchild and Fraps, 1944) and it occurs fairly regularly whenever the lay of an intraclutch egg is made to take place without the ovulation which normally accompanies it.

DISCUSSION

It is evident from these experiments, that in the presence of a functional ruptured follicle, lay occurs at the normally expected time after ovulation, or within the maximum limits expected of terminal eggs in normal birds, independently of the light-dark sequence. In contrast, the lay of eggs by birds lacking a functional ruptured follicle, and in which the possibility of control by ovulation is excluded (by excision of all maturing follicles in the rapid growth phase) is definitely correlated with light-dark sequence. While the correlation of lay with light is not absolute, it is of a sufficiently high order to leave little doubt that some agency, probably extra-ovarian, and capable of functioning in the absence of the ruptured or ovulating follicle, is involved in the process of oviposition of held eggs in the hen.

The first question raised by the existence of this agency is whether it is entirely independent of the normal process, and takes over as a sort of "double assurance" in default of the normally functional ovarian elements, or whether it is one of the participating factors in the normal process. While we cannot evaluate either of these possibilities at the present time, it is obviously simpler to assume that this agency is directly involved in the normal process of oviposition, and that the ruptured or ovulating follicle (or both acting together) determines the time of lay by acting through it; in the absence of the ovarian controls, the rhythmic alternations of light and darkness apparently take over this determination of the time of lay.

The second question is concerned with the nature of the extra-ovarian agency. It is of course tempting to suppose that it is, or is associated with, the anterior pituitary gland. This gland is known to be sensitive to light-dark sequences, through not perhaps as promptly as the results reported here indicate. Its control over various processes as well as its response to hormonal agents secreted by other organs are also too well known to call for review in this place. Finally, we have in hand some evidence, suggestive rather than definitive at this point, that the ruptured follicle is involved in the determination of the

time of ovulation of at least intra-clutch follicles, apparently through the anterior pituitary.

It should be pointed out that the posterior pituitary may also be involved. The efficacy of preparations from this gland in effecting lay in intact birds (Burrows and Byerly, 1942) also extends to birds lacking the ruptured follicle. The participation of the posterior pituitary in the process of oviposition has also been considered by Schooley and Riddle (1944) on the basis of the correlations between light and darkness and the time of lay in the intact ring-dove.

The identification of the extra-ovarian agency however, will be of greatest value when its relations to the normal process of lay can be demonstrated. Only insofar as it can aid in the design of experiments to answer this problem are we justified at the present time in supposing that the pituitary is involved in the process of lay.

SUMMARY

The ruptured follicle and all rapidly growing preovulatory follicles were removed from each of 48 hens. Twenty-one were then replaced under standard lighting (lights from 6:00 A.M. to 8:00 P.M.) and 27 under reversed lighting (lights from 4:00 P.M. to 6:00 A.M.), and observed for hour of lay. All except 4 birds retained their eggs, the majority of eggs in both groups being laid within 9 to 36 hours after the expected normal time. Seventeen of the retained eggs in each of the 2 groups were laid during lighted hours, the most frequent hours of lay in both groups occurring at 7 to 10 hours after the onset of light.

Seventeen out of 19 birds operated on similarly, but with the ruptured follicle left intact, laid their eggs at practically the normally expected time, under either the standard (6 birds) or reversed (13 birds) lighting.

It is concluded that an extraovarian, light-sensitive agent is involved in the process of lay.

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THE EFFECT OF VARIOUS GONADOTROPIC HORMONE PREPARATIONS ON THE BLOOD PLASMA ASCORBIC ACID OF SHEEP AND RABBITS[1]

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RECENTLY a number of observations have indicated a relationship of ascorbic acid to the endocrine control of reproduction. Experimental results have shown an effect of hormone preparations on ascorbic acid content of tissues and blood plasma, and conversely ascorbic acid has been reported to influence the response of animals to certain gonadotropic preparations.

Mosonyi (1936, 1937) observed a 30 to 40 per cent decrease in the ascorbic acid content of the livers and adrenals of guinea pigs after the administration of estrogens to females or androgens to males. Nespor (1937) found that there was first a rise and later a fall in the ascorbic acid content of the testes of young guinea pigs on the scorbutic diet after giving gonadotropic hormone. The simultaneous loss by other tissues indicated to him a utilization of ascorbic acid in the testes at the expense of other tissues. The ability of the testes to maintain their normal level of ascorbic acid when other tissues are depleted has been observed in selenium poisoned rats (Lardy and Moxon, 1942). Recently Erb and Andrews (1942, Andrews and Erb, 1942) observed that the gonadotropic hormone of pregnant mare's serum caused a decrease in the ascorbic acid of the blood plasma of cattle. Recovery to normal levels of blood plasma ascorbic acid occurred much more rapidly in castrated animals than in bulls or cows.

Giedosz (1938) was the first to report evidence that the administration of ascorbic acid may influence an animal's response to gonadotropic preparations. He found that in rabbits the hypertrophy of the ovaries produced by gonadotropic hormone was greater when 150–200 mg. of ascorbic acid was injected simultaneously. The ascorbic acid alone had no effect on the ovary size. Di Ció and Schteingart (1942) observed augmentation, by ascorbic acid, of the effect of gonadotropic hormone in both male and female rats but could not

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confirm Giedosz' observations in rabbits. Andrews and coworkers found ascorbic acid to be without influence on the response of chicks (Andrews and Erb, 1943) or rats (Almquist and Andrews, 1944) to gonadotropic hormone. They likewise found administration of the hormone to have no effect on the blood plasma ascorbic acid of the chick.

In the present study the effect of various gonadotropic hormone preparations on the blood plasma ascorbic acid of sheep and rabbits was investigated. For the sake of brevity BPAA will hereafter be employed to designate "blood plasma ascorbic acid."

METHODS AND MATERIALS

Blood samples were drawn, from the jugular vein of ewes and from the marginal ear vein of the rabbits, into oxalated centrifuge tubes. The samples were placed on ice in the dark and were brought to the laboratory and analyzed within an hour. The photometric method of Mindlin and Butler (1938) was used for the determination of the ascorbic acid.

The hormone preparations were as follows: *Gonadotropic substances of pregnant mare's serum*[2] was used as a whole serum which had been kept frozen. Its potency was 80 I.U./cc. The preparation will hereafter be referred to as PMS. *Follicle stimulating extract of sheep pituitary*[3] was prepared by the "trypsin digestion" method of McShan and Meyer (1940). When injected subcutaneously into 21 day old rats (Sprague-Dawley strain) twice daily for four and one half days (total dosage equivalent to 500 mg. original desiccated pituitary powder) it produced ovaries weighing approximately 80 mg. This extract (hereafter referred to as FSH) produced only follicular development. *The unfractionated pituitary extract*[3] ("B" fraction of McShan and Meyer (1943)) was of such potency that an assay of 50 mg. by the method described under (2) above produced ovaries weighing an average of 78 mg. This material produced both follicular development and luteinization.

RESULTS

Effect of PMS. The effect of the subcutaneous injection of 5 cc. (400 I.U.) of the PMS on the BPAA of anestrual ewes is shown in table 1. The BPAA had decreased in all ewes 8 hrs. after administration of the hormone (average decrease 23 per cent) and, with the exception of 2 samples collected 9 days after the first injection, remained low for a period of 16 days. A second injection (400 I.U.) of PMS was given on the 16th day and 24 hours later all of the ewes showed a transient rise in BPAA. It is of interest that the two ewes (Nos. 73 and 81) which had the largest increase in BPAA following the second injection also were in heat 48 hours after this treatment. Ewe No. 81 was still in heat on the 3rd day after the treatment. Sub-

[2] The pregnant mare's serum was kindly furnished by Upjohn Co., Kalamazoo, Michigan.

[3] We are indebted to Dr. McShan of the Department of Zoology, University of Wisconsin for these preparations.

TABLE 1. EFFECT OF PMS ON THE ASCORBIC ACID CONTENT OF EWES' BLOOD PLASMA

Ewe no.	Blood plasma ascorbic acid (mg./100 cc.)							
	Control value*	Time after administration of PMS					2nd dose	
		8 hrs.	23 hrs.	5 days	9 days	16 days	24 hrs.	3 days
68	.72	.55	.53	.49	.73	.49	.62	.51
73	.61	.46	.47	.44	.34	.39	.73	.56
81	.82	.60	.61	.61	.68	.59	1.03	.70
84	.73	.59	.63	.61	.84	.53	.95	.45
Ave.	.72	.55	.56	.54	.65	.50	.83	.56

* Level of ascorbic acid immediately before the first injection of PMS. 400 I.U. of PMS injected subcutaneously on 0 and 16th day.

sequent treatment of these ewes in connection with another experiment prevented further analyses.

Administration of the same quantity of normal horse serum to another ewe caused no depression of the BPAA.

In table 2 are shown the effects of administering 5 cc. (400 I.U.) of PMS in a single subcutaneous dose to each of three pseudopregnant and three anestrual female rabbits. All of the does showed a decrease of BPAA from the pre-treatment level. The recovery could again not be followed in these animals because of subsequent treatment.

Effect of FSH. The subcutaneous injection of FSH brought about anomalous changes in the BPAA in three experiments with rabbits

TABLE 2. EFFECT OF PMS (400 I.U. SUBCUTANEOUSLY) ON THE ASCORBIC ACID CONTENT OF RABBITS' BLOOD PLASMA

Rabbit no.	Blood plasma ascorbic acid (mg./100 cc.)	
	Before treatment	12.5 hrs. after treatment
184*	.35	.09
118*	.51	.14
153*	.64	.35
80†	.66	.25
96†	.51	< .01
156†	.57	.02
Ave.	.54	.14

* These does were in heat and mated to vasectomized bucks 5 days before receiving the PMS injection.
† Anestrual does.

and one with sheep. In experiments A, B, and C (table 3) 0.5 gm. equivalent of FSH was injected into each rabbit on each of 5 successive days. Blood samples were collected before treatment and again on the fifth day of the experiment. The average BPAA of the does in each lot increased after the treatment. The increase was not

statistically significant for lots A and B but was highly significant ($P = 0.01$) for lot C.[4]

It appears that the ability of does to respond to this treatment may have been related to their original BPAA concentration. The original levels of BPAA in the does in experiments A and B were significantly[3] lower ($P = <0.02$) than in the does used in experiment C. These low levels of BPAA may have been the result of an inadequate intake of vitamin A in the rations, for experiments A and B were carried out in February when the content of hays has usually reached a low level. Experiment C was carried out the following June. It has been demonstrated that the ability of cattle (Boyer *et al.*, 1942) and rats (Sutton *et al.*, 1942) to maintain normal levels of BPAA depends on an adequate intake of vitamin A.

TABLE 3. EFFECT OF FSH ON THE ASCORBIC ACID CONTENT OF RABBITS' BLOOD

Lot A—Estrual does		Lot B—Pseudopregnant does		Lot C—Estrual does	
Blood plasma ascorbic acid (mg./100 cc. plasma)					
Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
.19*	.08*	.16	.30	.43	.28
.14	1.24	.25	.44	.27	.40
.15	.13	.14	1.24	.27	.50
.16	.19	.13	.18	.53	.86
.25	.21	.45	.48	.40	.53
.08	.12	.20	.08	.50	.51
.12	.18	.13	.15	.20	.42
.09	.14	.20	.32	.25	.40
.19	.44	.30	.30	.24	.50
				.32	.59
				.04	.20
Ave. .15	.30	.22	.39	.31	.47

Treatment described in the text.
* Each pair of values (before and after treatment) represents an individual doe.

Effect of FSH and unfractionated pituitary extract in ewes. The data in tables 4 and 5 were obtained from ewes treated in connection with another investigation (Casida, Dutt and Meyer, unpublished). The experimental procedure allowed observation of the effect of these gonadotropic preparations when administered at various stages of the estrual cycle.

Each ewe in the group treated with FSH was given one subcutaneous injection of 7.5 gm. equivalents of FSH extract on each of 4 successive days beginning on either the 4th, 8th or 12th day of the estrual cycle. Blood samples were collected before, 8 hours after and 4 days after the first injection. Following treatment with FSH, the greatest

[4] We are indebted to Miss Virginia Beal of the Experiment Station Statistical Service for this analysis by the "Analysis of Variance" technique.

percentage decrease occurred in lot 3 where the ewes had a higher original level of ascorbic acid. The levels finally reached were about the same for all lots. For the group of FSH treated ewes as a whole,

TABLE 4. EFFECT OF FOLLICLE STIMULATING HORMONE AT VARIOUS STAGES OF THE ESTRUAL CYCLE ON THE BLOOD PLASMA ASCORBIC ACID OF EWES

Lot	Days after estrum	Ewe no.	Plasma ascorbic acid (mg./100 cc.)		
			Control value	Time after treatment with FSH	
				8 hours	4 days
1	4	21	.46	.38	.33
		13	.68	.53	.35
		24	.58	.50	.22
		Ave.	.57	.47	.30
2	8	95	.48	.53	.22
		33	.62	.53	.30
		10	.49	.37	.57
		92	.49	.36	.40
		Ave.	.52	.45	.37
3	12	7	.72	.53	—
		30	.76	.59	.39
		31	.76	.47	.49
		1	.70	.40	.18
		Ave.	.74	.49	.35

TABLE 5. EFFECT OF UNFRACTIONATED PITUITARY EXTRACT AT VARIOUS STAGES OF THE ESTRUAL CYCLE ON THE BLOOD PLASMA ASCORBIC ACID OF EWES

Lot	Days after estrum	Ewe No.	Plasma ascorbic acid (mg./100 cc.)		
			Control value	Time after treatment with pit. ext.	
				8 hours	4 days
1	4	11	.20	.00	.22
		16	.63	.52	.61
		100	.84	.46	.52
		6	.48	.42	.18
		Ave.	.54	.35	.38
2	8	8	.57	.62	.46
		14	.62	.55	.58
		93	.58	.38	.51
		Ave.	.59	.52	.52
3	12	2	.79	.64	.35
		15	.52	.58	.35
		98	.68	.78	.51
		17	.77	.63	.79
		Ave.	.69	.66	.50

the decreases in BPAA were statistically significant[4] for both the 0–8 hour and 0–4 day interval (P, in both cases, =0.01).

Each ewe in the group treated with unfractionated pituitary extract received 4 successive daily subcutaneous injections of 1.2 gm.

equivalents of the extract. This group of ewes was likewise divided into 3 lots which received their first injection on the 4th, 8th and 12th day respectively, of the estrual cycle. While the changes in BPAA were not as dramatic, nor as consistent, as in the case of FSH treatment, for the group as a whole, a probably significant decrease had occurred 8 hours after treatment ($P = 0.05$) and a significant decrease ($P = 0.01$) had occurred by the 4th day.[5]

Inspection of these results indicates that, at the dosage used in this experiment, FSH was more effective in reducing BPAA than was the unfractionated extract. However, the differences between the decreases produced by FSH and by unfractionated extract were not statistically significant.

Effect of stage of estrual cycle on BPAA. It is possible that the BPAA level of ewes varies with the stage of the estrual cycle. In the experiments shown in tables 4 and 5 the control levels of BPAA of ewes on the 12th day of the cycle were found to be higher ($P = 0.05$) than in similar ewes on the 4th and 8th day.[3] In this connection it is of interest to note that in the experiment shown in table 1 the ewe (No. 81) remaining in heat on the 3rd day after the second injection of PMS had a high BPAA value. It has been shown previously (Phillips and coworkers, 1941) that cows show an increase in BPAA at the time of estrus.

DISCUSSION

Preparations of gonadotropic hormones from pregnant mares' serum have been found to depress the BPAA of cows and bulls (Erb and Andrews, 1942), steers (Andrews and Erb, 1942), and of ewes and rabbits (this report). No such effect was found in chicks (Andrews and Erb, 1943) or rats (Almquist and Andrews, 1944), (Meyer, Phillips and coworkers, unpublished). That there are species differences in the responses to this preparation is evident.

It has now been found that the administration of other gonadotropic hormone preparations also may affect BPAA. And species differences in response to one of these were likewise observed. FSH was effective in reducing BPAA in ewes, yet it increased the BPAA in does. Failure of the same FSH preparation to increase BPAA to a level of statistical significance in two other experiments with does may have been the result of their original low level of BPAA.

SUMMARY

The subcutaneous administration of a preparation of pregnant mare's serum (400 I.U.) was effective in reducing the blood plasma ascorbic acid in ewes. The average decrease was 23 per cent of the original level and the ascorbic acid concentration remained essentially unchanged for 16 days. The administration of a second dose (400

[5] Tested by Student's method of analysis for correlated samples (Paterson, 1939).

I.U.) of pregnant mare's serum brought about a transient rise in the blood plasma ascorbic acid level. The subcutaneous administration of 400 I.U. of pregnant mare's serum decreased the blood plasma ascorbic acid of both pseudopregnant and anestrual rabbits. A follicle stimulating extract prepared from sheep pituitary glands was effective in reducing the blood plasma ascorbic acid of ewes when administered on either the 4th, 8th or 12th day of the estrual cycle. The same preparation increased the blood plasma ascorbic acid significantly ($P = 0.01$) in one group of rabbits but was without significant effect on 2 groups of rabbits having an initially low level of ascorbic acid. The relation of the nutritive state of these does to their response to the hormone is discussed. An unfractionated pituitary extract (containing both follicle stimulating and luteinizing activity) depressed the blood plasma ascorbic acid of ewes but probably with less certainty than the follicle stimulating extract. The normal blood plasma ascorbic acid level of ewes appeared to be higher on the 12th, than on the 4th or 8th day of the estrual cycle.

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THE EFFECT OF INSULIN ON THE URINARY EXCRETION OF SODIUM, CHLORIDE, NITROGEN AND GLUCOSE IN NORMAL RATS

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IN PRELIMINARY studies (unpublished) of the effect of insulin on adrenal steroid diabetes in normal rats it was noted that insulin alone affected the quantities of certain crystalloids present in the urine. The present study was undertaken in order to determine the tolerance of normal rats to insulin under certain experimental conditions and to evaluate any accompanying changes in the excretion of sodium, chloride, nitrogen and glucose. It was found that states of insulin hypoglycemia were followed by an increased sodium and chloride loss, an increased excretion of non-protein nitrogen and inhibition of growth or a definite loss of weight. Following the abrupt withdrawal of large doses of insulin there was a temporary period of hyperglycemia.

METHODS

Male rats of the Sprague-Dawley strain were maintained on a diet of Purina dog chow until they reached a weight of 300 to 350 grams. They were

TABLE 1

Constituent	grams
Cellu Flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Wheat germ oil	10
Mazola Oil	10
Cod liver oil	10
Dried yeast (Pabst)	100
Egg albumin (Merck)	160
Starch	500
Dextrin	250
Sucrose	250
Water to make total volume of	2000 cc.

then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:00 A.M.) and late afternoon (4:30 to 5:00 P.M.). The technique of force-feeding and the high carbohydrate diet used were modifications of those described by Reinecke, Ball and Samuels (1939). The diet was made according to Table I. During the period of adaptation to force-feeding the diet was administered in small amounts to

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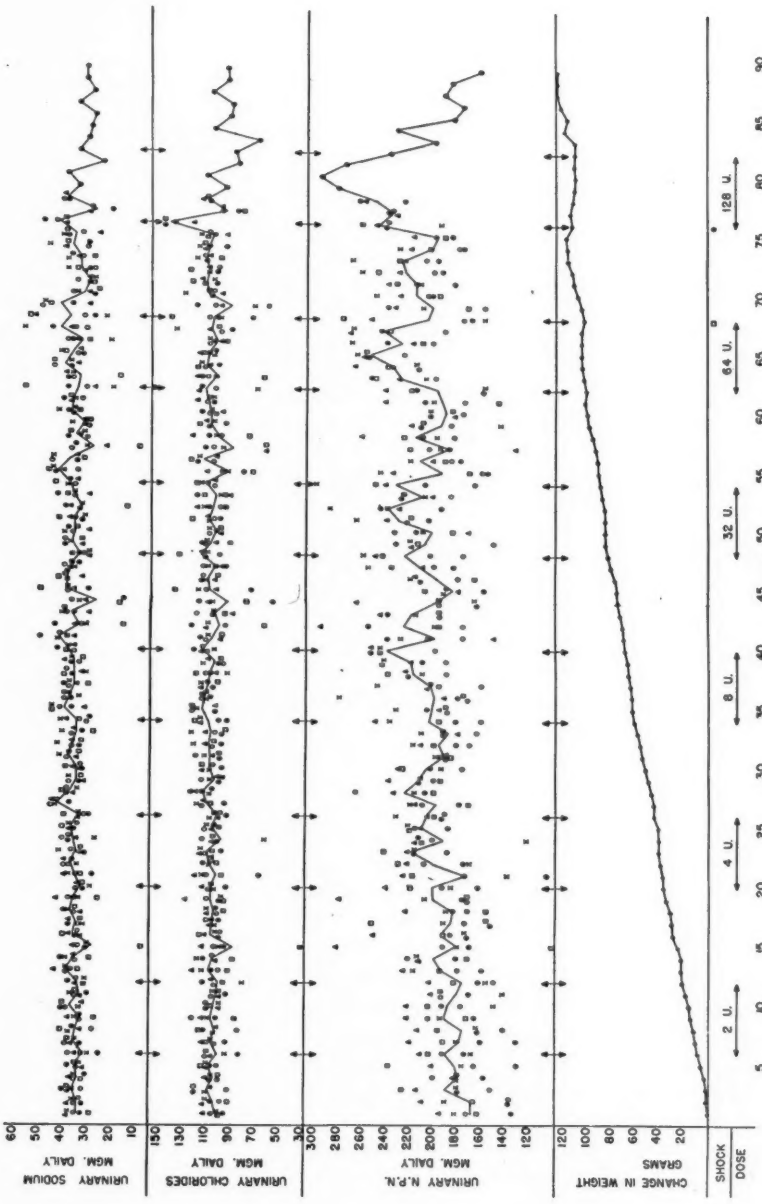

FIG. 1. The effect of regular insulin on body-weight and on urinary non-protein nitrogen, chlorides and sodium. Each symbol represents an individual animal. The arrows represent the times at which insulin was either started or withdrawn.

prevent the development of "food shock." The animals were brought to a full feeding on the fifth day. Each rat received 26 cc. of diet per day which contained approximately 15 grams of available carbohydrate.

The injections of insulin were begun after a minimum control period of ten days and were made subcutaneously twice daily immediately after each feeding. Regular insulin and protamine zinc insulin (Lilly) were the preparations used.

Twenty-four-hour specimens of urine were collected at the same hour each day and preserved with thymol. The following methods of analysis were used: Blood glucose, Miller and Van Slyke (1936); urinary glucose, Benedict (1911); urinary chloride, Schales and Schales (1941) and urinary sodium, Butler and Tuthill (1931). The determination of non-protein nitrogen was by the micro-Kjeldahl procedure as follows: Proteins were precipitated as salts of tungstic acid by the Folin-Wu procedure. The organic matter was oxidized by sulfuric acid and hydrogen peroxide. The ammonia was distilled off into a standard acid solution and titrated with standard base.

EXPERIMENTS AND RESULTS

Experiment 1 (figure 1), was a study of the tolerance of normal rats to regular insulin and of some of the metabolic changes. Five rats were each treated in an identical manner. Following an initial control period of ten days, the seven-day periods of insulin injection were alternated with seven-day control periods. The dosages used were 2, 4, 8, 32, 64 and 128 units of regular insulin daily. Four of the rats died of insulin shock during the administration of 128 units of insulin per day and one of the rats survived all of the injections of insulin.

Experiment 2 (figure 2) was a study of the tolerance of the normal rat to protamine zinc insulin and of some of the metabolic changes. It was carried out in a manner identical to that of Experiment 1. The dosages tested were 2, 3, and 4 units of protamine zinc insulin daily. Two of the five animals died of insulin shock during the administration of 3 units per day and two animals died during the administration of 4 units per day. One animal survived the administration of 4 units per day but died on the second day following the withdrawal of insulin, apparently of diabetic shock.

Tolerance to insulin. Under the conditions of these experiments there was a striking difference in the tolerance of force-fed rats to regular insulin and protamine zinc insulin. The largest dose of regular insulin tested was thirty-two times greater than the largest dose of protamine zinc insulin. This is probably due to the difference in the duration of action of the two forms of insulin. The absorption of glucose from the gut following feeding was of sufficient duration to compensate for the blood-sugar lowering effect of regular insulin in all but massive doses whereas the blood sugar lowering effect of protamine zinc insulin persists beyond the period of carbohydrate absorption.

Blood glucose. Samples of tail blood were taken at various times

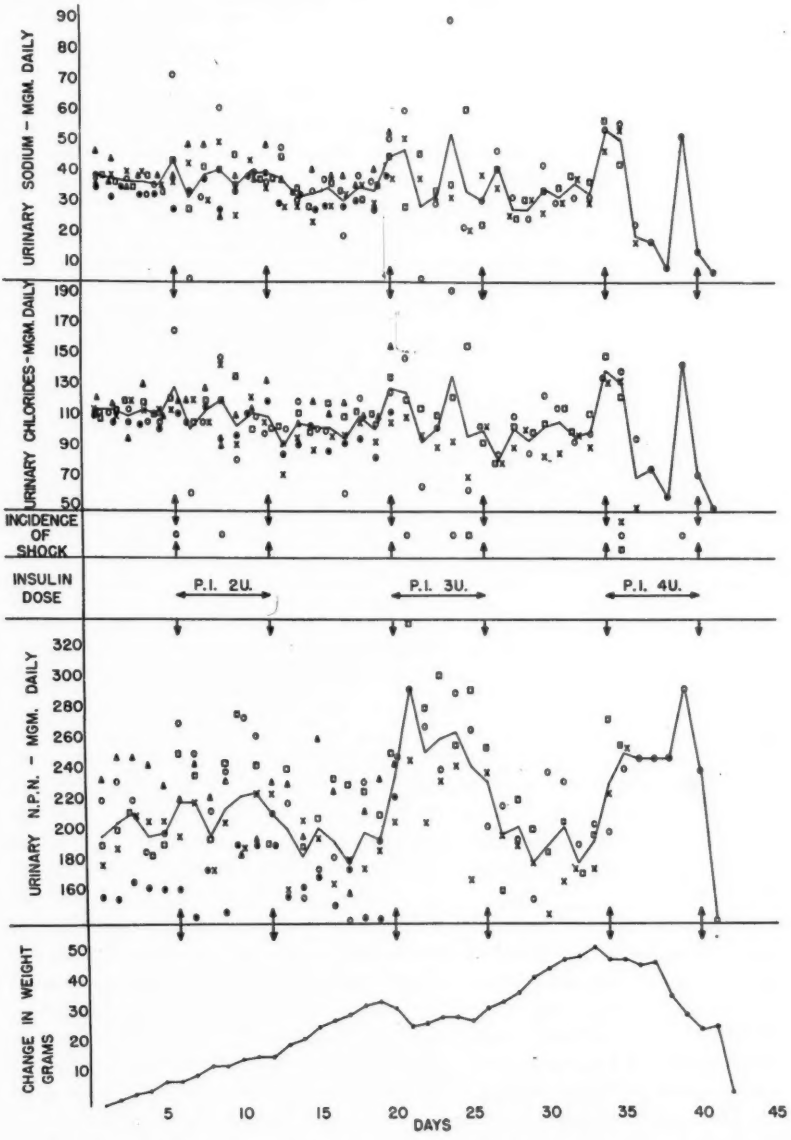

FIG. 2. The effect of protamine zinc insulin on body-weight and on urinary non-protein nitrogen, chlorides and sodium. Each symbol represents an individual animal. The arrows represent the times at which insulin was either started or withdrawn. It will be noted that much of the apparent irregularity in the values for chlorides and for sodium is related to either the beginning of the administration of insulin or to the occurrence of insulin shock. Increased excretion was usually followed immediately by compensatory retention.

during the experiments. The first sample was obtained just prior to the morning feeding (sixteen hour fast) and additional samples were obtained at two, four, six and eight hours following the morning feeding. It is important to note that all animals tolerated the administration of the high carbohydrate diet without the development of hyperglycemia prior to the administration of insulin. When regular insulin was administered in doses up to 8 units per day the values for blood glucose remained within normal limits following feeding. With doses

TABLE 2. VALUES FOR BLOOD GLUCOSE IN NORMAL RATS TREATED WITH INSULIN

Rat	Form of insulin	Units dose insulin	Values for blood glucose, mgm. %				
			16 hour fast	Hours following morning feeding			
				2	4	6	8
1004		0	93	111	99	109	109
	regular	4	111	119	123	126	126
		8	111	80	120	111	117
		32	115	83	61	75	67
		64	163	93	73	66	63
		128	115	82	75	51	55
	1st day after stopping insulin	0	150	198	189	190	177
602		0	99	123	111	117	125
	protamine	3	61	114	105	99	53
	protamine	4	18	105	96	120	123
	2nd day after stopping insulin		Blood glucose not determined but 2138 mgm. of glucose were excreted in the urine.				

of 32 units and higher there was a depression of blood glucose following feeding but the values after a sixteen-hour fast were higher than normal. In rat 1004 the values for blood glucose the day following withdrawal of the 128 unit dose remained at hyperglycemic levels following feeding and during fasting but the animal did not excrete glucose.

The animals treated with protamine zinc insulin showed little or no depression of the level of blood glucose during the first few hours following feeding but it later fell to hypoglycemic levels and remained low until the next feeding. Rat 602 developed glycosuria and died, apparently in diabetic shock during the second day following the withdrawal of 4 units per day of protamine zinc insulin. No samples of blood were obtained during this period.

Data illustrating the changes in blood glucose are summarized in Table 2.

Insulin shock. The incidence of insulin shock has been illustrated in Figures 1 and 2. All of the animals were carefully observed for signs of shock during the day, but if shock and subsequent recovery occurred during the night and prior to the morning feedings it would

not have been noted. As indicated by the data on blood glucose, severe hypoglycemia and shock occurred much less frequently among animals given regular insulin than among animals given protamine zinc insulin.

Urinary glucose. All of the animals failed to excrete glucose during the control periods. Among the hundreds of normal animals to which we have force-fed high carbohydrate diets during the past five years we have never observed a case of spontaneous glycosuria or hyperglycemia. It may be of some significance that normal rats treated with insulin may occasionally excrete small amounts of glucose. Rat 605 excreted 185 mgm. of glucose during treatment with 128 units of regular insulin on the day prior to death. Rat 505 excreted 165 mgm. of glucose during treatment with 4 units per day of protamine zinc insulin on the day prior to death. Rat 602 excreted 139 mgm. of glucose on one day during treatment with 3 units per day of protamine zinc insulin, 71 mgm. of glucose on one day during treatment with 4 units per day and on the second day following the withdrawal of this dose the animal excreted 2138 mgm. of glucose (this sample represented a fifteen-hour period) and died following the development of symptoms of dehydration and shock which characterize the sudden exacerbation of a diabetic state in the rat. These data are not included in the figures.

Body-weight. The average changes in weight are included in Figures 1 and 2. The daily administration of 2, 4, 8, and 32 units of regular insulin had little or no effect on the rate of growth but there was inhibition of growth during the administration of 64 and 128 units daily. The administration of 2 units per day of protamine zinc insulin did not have any clear effect on the rate of growth but doses of 3 and 4 units per day caused definite inhibition of growth and a loss of weight.

Non-protein nitrogen. The data on non-protein nitrogen are included in Figures 1 and 2. The daily administration of 2, 4, 8 and 32 units of regular insulin had no significant effect upon the urinary nitrogen but there was some increase during the administration of 64 and 128 units daily. The effect of protamine zinc insulin was more striking. There was a small but possibly insignificant increase in the excretion of non-protein nitrogen during the administration of 2 units per day and very striking increases during the administration of 3 and 4 units per day. It was noted that the values for individual animals were always high following insulin shock.

Urinary sodium and chlorides. The data on the urinary sodium and chlorides are given in Figures 1 and 2. The administration of 2, 4, 8 and 32 units of regular insulin per day did not produce a significant change in any of the animals. When 64 units per day was administered rat 608 showed a significant increase of both sodium and chlorides during the first day of injection. When 128 units per day

were administered the four surviving rats showed a significant increase in the excretion of chlorides and a less marked increase in the excretion of sodium. Increases in the excretion of chlorides were found to occur during the administration of protamine zinc insulin in amounts of 2, 3 and 4 units per day. Increases in the excretion of chlorides and sodium were associated either with the beginning of insulin administration or with the occurrence of insulin shock. An increase above normal was usually followed by compensatory retention. Changes in the level of urinary sodium were usually, but not always, accompanied by similar changes in the excretion of chlorides.

DISCUSSION

The changes in growth and in the excretion of non-protein nitrogen, sodium and chloride observed in these experiments were associated with states of hypoglycemia and shock. They may not represent primary effects of the normal hormonal actions of insulin but rather they may be secondary to insulin shock or other effects of greater than physiologic amounts of insulin.

It is well established that under certain experimental conditions the administration of insulin to non-diabetic animals (Janney and Shapiro, 1926; Mirsky, 1938; Mackay, Barnes and Berman, 1939) and patients results in the sparing of protein. In the present experiments there was no increase in the rate of growth or a decrease in the level of urinary nitrogen during the administration of insulin. On the contrary when the dosage of insulin was increased sufficiently to cause metabolic changes there was an increase in the amounts of nitrogen excreted and either a slowing of growth or a definite loss of weight. This effect of large doses of insulin in increasing protein catabolism was observed by Sokhey and Allan (1924) in normal dogs and by Kiech and Luck (1928) in normal rats. There is no doubt that experimental conditions can be set up under which the administration of insulin favors protein anabolism and that under other experimental conditions it causes, possibly secondarily, an increase in the rate of catabolism of protein. The absence of an anabolic effect when small doses were administered in these experiments may indicate that the retention of nitrogen had already been going on at a maximum rate in these growing animals fed a low protein diet. The increase in urinary nitrogen during the administration of large doses of insulin may represent an increase in gluconeogenesis from protein, stimulated in some unknown manner to occur as a compensatory response to the low level of blood glucose. Other mechanisms may have been involved.

The occurrence of hyperglycemia or a decrease in carbohydrate tolerance following a period of insulin hypoglycemia in animals or patients is a well known phenomenon, first observed by Wilder, Smith and Sandiford (1932) and by Paul, Clark and Gibson (1932). The occurrence of glycosuria following the withdrawal of insulin has

been reported by Clark, Gibson and Paul (1935) who demonstrated transitory hyperglycemia and glycosuria following the discontinuation of insulin in non-diabetic patients and by Mirsky *et al.*, (1942) in studies on the dog. There is evidence (Haist, Campbell and Best, 1940) to support the assumption that a major factor in the production of this phenomenon is the suppression of the secretory activity of the pancreatic islet tissue in the presence of an excess of exogenous insulin. It is possible that other compensatory physiologic mechanisms are activated by an excess of insulin and may contribute to the production of hyperglycemia following the sudden withdrawal of insulin.

Attempts have been made to reproduce in six other rats the diabetic state noted in rat 602 after the withdrawal of insulin. In two additional cases a slight glycosuria has been observed. Observations on the amounts of glucose in the blood have provided an explanation for the failure to obtain a more convincing confirmation. We have failed to keep the level of blood glucose at low levels throughout the day. It was anticipated that a mixture of regular insulin and of protamine zinc insulin might accomplish this purpose but these attempts were also unsuccessful. These studies have not been exhaustive and certain refinements in technique remain to be tested.

It is probable that the death of rat 602 was related to the diabetic state although the amount of glucose which it excreted prior to death was not large. The onset of glycosuria began during the day of its death and only a fifteen-hour sample was obtained which contained more than two grams of glucose. We have many similar observations of death in rats following the sudden exacerbation of a diabetic state by the administration of the 11, 17-oxygenated adrenal steroids or by diethylstilbestrol. When the rate of onset of glycosuria is slow the animal is able to make a more adequate adaptation to the metabolic defects and a much more severe diabetic state can be tolerated.

The occasional excretion of small amounts of glucose by normal rats given large doses of insulin is not a phenomenon occurring in untreated normal animals. In our experience even these small amounts of urinary glucose never occur spontaneously. In the early literature on the effects of insulin there were many reports of an initial hyperglycemia following the administration of large doses to animals or patients. This was shown by Geiling and de Lawder (1930) to be due to impurities present in amorphous preparations of insulin. We are unable to give a full explanation for the occurrence of glycosuria in these experiments.

It has been shown in these experiments that large doses of insulin cause, probably secondarily, increases in the amounts of sodium and chloride excreted in urine. Our interest in such effects has to do with their possible relationship to the function of the adrenal cortex. As noted by Thorn, Engel and Lewis (1941) the cortical steroids 17-hydroxycorticosterone and 17-hydroxy-11-dehydro-corticosterone

cause an increase in the urinary excretion of sodium and chlorides in normal animals. In the studies by Lewis *et al.* (1942) it was shown that anoxia resulting in acapnia caused an increase in the excretion of sodium and chloride in intact animals but not in adrenalectomized animals. The hypothesis was offered that the increased excretion of sodium and chloride was due specifically to the increased secretion of the 11, 17-oxygenated cortical steroids. It has been reported by Britton and Silvette (1937) that a lowering of the levels of sodium and chloride in the blood serum follows the occurrence of other forms of stress in non-adrenalectomized animals. The adrenal cortices are known to hypertrophy in response to anoxia, large amounts of insulin or any other form of severe stress. The increased excretion of sodium and of chloride in all such cases might be explained by the hypothesis that the effect is due specifically to the increased secretion of the 11-, 17-oxygenated cortical steroids. Ingle (in press) has pointed out that a second hypothesis is tenable. It is possible that a number of non-specific stimuli may cause a temporary increase in the urinary excretion of sodium and chloride. The effect of large doses of the 11, 17-oxygenated cortical steroids may only represent these non-specific stimuli in addition to anoxia, insulin shock, severe surgery, etc. and may not be the mediating factor. An increase in protein catabolism may or may not be an essential condition. As reported by Ingle and Sheppard (1944) the effect of the 11, 17-oxygenated cortical steroids on sodium and chloride balance is temporary and is followed by compensatory retention. To test these hypotheses would require the use of adrenalectomized animals treated by a constant intake of optimal amounts of cortical extracts. Such experiments have not been carried out. Our consideration of this hypothesis does not represent an endorsement of it over the hypothesis advanced by Lewis *et al.* (1942). At the present time we are only concerned with listing the possible explanations.

SUMMARY

The tolerance of normal, force-fed, male rats to regular insulin and to protamine zinc insulin was studied in these experiments. Small doses of insulin did not produce any changes from normal. States of insulin hypoglycemia and shock were associated with inhibition of growth or a definite loss of weight, an increased sodium and chloride loss which was followed by compensatory retention, an increased excretion of non-protein nitrogen and an occasional excretion of small amounts of glucose in the urine. Following the withdrawal of large doses of insulin there was a temporary period of hyperglycemia and in one rat glycosuria appeared.

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IODINE IN BLOOD AND THYROID OF MAN AND SMALL ANIMALS

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IN STUDIES of iodine metabolism, particularly those involving the blood and the thyroid, it is becoming important to work with small samples. This is especially true in observations on small animals; but in the clinic, e.g., in pediatric studies, it is also essential that blood "hormonal" iodine (Salter, 1940) be determined without drawing too much human blood. This paper describes a method which will give duplicate analyses on 2 cc. of serum, and is adapted to standard photoelectric colorimeters such as are available in many laboratories at the present time.

It is now well established that human thyroid function can be measured satisfactorily by serum iodine analysis, even when the serum is transmitted long distances in simple containers (Salter, Bassett and Sappington, 1941; Riggs, Gildea, Man and Peters, 1941). Normally the concentration of the "hormonal" iodine is approximately 5 micrograms per cent, or 0.05 micrograms per cc. in man. Although the normal limits must be established arbitrarily in each laboratory, approximately 0.04 and 0.08 micrograms per cc. are reasonable limits of normality for practical purposes. In order to measure such a small aliquot of iodine, advantage was taken of the work of Sandell and Kolthoff (1937) who studied the reaction of iodine as a catalyst upon the following reaction:

$$\underset{\text{(Yellow)}}{2Ce^{++++}} + H_2AsO_3^- + H_2O = \underset{\text{(Colorless)}}{2Ce^{+++}} + H_2AsO_4^- + 2H^+$$

Indeed, it was suggested by Chaney (1940) that this reaction might be applied to biological materials.

Although this catalytic method works well in pure solutions, it has been difficult to apply it to biological materials, like blood, muscle or thyroid. The chief difficulty has consisted in selecting the sample and in preparing it for analysis by some appropriate ashing or oxida-

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tion procedure. Elsewhere there is published (Sappington, Halperin and Salter, 1944) a method for use in the so-called pharmacological range, such as might be encountered in pharmacotherapy or in studies of the thyroid gland itself. The present communication is concerned with the determination of iodine in blood and tissues near the physiological range of concentration.

ANALYTICAL PROCEDURE

The method involves a preliminary precipitation of the protein-bound iodine (Bassett, Coons and Salter, 1941) and separation of the soluble iodide (Salter, 1940). These two moieties are treated separately. After preliminary ashing, the iodine in the ashed material is determined colorimetrically. The following reagents are needed:

Water. Distilled water is prepared by redistilling ordinary once-distilled water in an all-glass apparatus containing about 20 grams of sodium hydroxide in a 5-liter flask. The initial distillate is tested and discarded until it becomes neutral to methyl red. It should be freshly boiled to eliminate peroxide.

Sodium carbonate, anhydrous granulated. (Mallinckrodt, A.R.). This material may be weighed to the nearest milligram for each analysis. It is more conveniently delivered volumetrically as 1 cc. of a 21.2 per cent (by volume) aqueous solution.

Sodium hydroxide (Mallinckrodt, A.R.). A 10 per cent solution (by volume) accurate within 1 per cent.

Sodium hydroxide, C.P. An 8 per cent aqueous solution.

Sodium bicarbonate, C.P. An 8.4 per cent aqueous solution.

Potassium nitrate, A.R. Crystals, finely powdered.

Low-nitrogen sulfuric acid (Mallinckrodt, C.P.). From this stock a 7.0 Normal aqueous solution is prepared.

Standard solution of potassium iodide. Per liter this stock aqueous solution contains 261.6 milligrams of potassium iodide.

Working standard solution of potassium iodide. This solution contains 1.0 cc. of the stock solution diluted to 1 liter with distilled water. This solution should be prepared freshly every 48 hours, and kept cool in the dark.

Ceric ammonium sulfate, 0.05 N. Per liter this stock solution contains 29.82 grams of ceric salt, dissolved in 1.6 Normal sulfuric acid. It must be standardized occasionally against 0.1 Normal arsenious acid.

Arsenious acid, 0.1 Normal (Mallinckrodt, A.R.). This contains per liter 4.947 grams of AS_2O_3 in 0.01 Normal sulfuric acid.

Separation of "hormonal" iodine from iodide. For the determination of "hormonal" iodine a pyrex test tube 1.3 cm. in internal diameter and 11 cm. long is provided with graduations at 5.0 cc. and 6.0 cc. Into this tube is measured 1.0 cc. of serum, followed by 2. cc. of 0.02 Normal acetic acid. Water is then added to the 5.0 cc. mark, and the liquid is made homogeneous. It is then stood in a beaker of cold water which is heated to boiling over the course of ten minutes. During this time frequent stirring prevents the coagulation of the curd of protein in a large lump, and produces a grossly flocculent but easily workable precipitate. This precipitate contains the "hormonal" iodine which is separated by gentle centrifugation. The clear supernatant

liquor is set aside for determination of iodide. Finally, the curd is washed twice with 5 cc. of 0.002 N acetic acid, by means of centrifugation.

To the curd at the bottom of the test tube is added 0.9 cc. of 8 per cent sodium hydroxide (by volume), and the tube is digested in a bath of boiling water, with occasional shaking, until all solid matter is dissolved. Thereafter, digestion is continued for thirty minutes to three hours. A fine glass rod may be used to knead the soda solution into the curdy mass of protein, and the rod is wiped carefully against the inside of the test tube on withdrawal. Next, 1.8 cc. of 8.4 per cent sodium bicarbonate solution is added. Finally, after much of the water has been removed by evaporation, 40.0 mg. of potassium nitrate, A.R. is added to this solution. Note that the alkali solutions should be delivered accurately with a small pipette, so that the total electrolytic system contains exactly 4 milliequivalents of sodium plus potassium.

For the evaporation, the tube is clamped in an inclined position, resting in a bath of warm water at about 50°C., and a circulation of iodine-free air is maintained through a small capillary which dips into the test tube. As the excess of liquid evaporates, the tube is rotated occasionally so that the dry film of organic material may be smeared evenly over the surface of the tube. Alternatively, the tube may simply rest in an oven at 95°C. overnight. In this way much bother can be eliminated because digestion and evaporation occur in one operation.

Total iodine. If no separation is desired, 0.50 or 1.0 cc. of serum may be placed in the tube, the sodium carbonate (212 mg.) added, and the liquid evaporated to dryness. Probably sodium hydroxide in equivalent, stoichiometric amount is also effective.

Inorganic iodine, naturally occurring. From the supernatant liquor separated by centrifugation, a convenient aliquot is measured, e.g., 3.5 cc., and placed in a second graduated pyrex test tube for oxidation. To this solution is added 212 mg. of sodium carbonate, and the liquid is evaporated off prior to ashing. Care should be taken that the organic matter is distributed in a thin film over the inner surface of the combustion tube.

Oxidation of organic materials. Each tube is next placed, with several other tubes, in a small electric muffle furnace. In this work the furnace manufactured by the Will Corporation, Rochester, New York, equipped with a pyrometer ranging up to 1100°C., was used. At first, heating must be undertaken cautiously, lest entrapped steam explode and blow the contents of the tube out into the furnace. Once the material is sufficiently dried, however, the temperature can be elevated rapidly to 650°C. It is maintained for three hours between 600°C. and 650°C. This corresponds to "high heat" on the rheostat control. For anhydrous sodium iodide, the melting point is 651°C. and the boiling point 1300°C. For the dihydrate ($NaI \cdot 2H_2O$) the corresponding values are, respectively, 752 and 1350.

The contents of the oven are then allowed to cool rapidly and the tube is removed.

Determination of iodide after ashing. To the contents of the tube is added 2.0 cc. of distilled water followed by the cautious addition of 1.5 cc. of 7 Normal sulfuric acid. Additional water is added until the total volume is 6.0 cc., and the tube is centrifuged rapidly if necessary. Next, from this solution two samples respectively of 3.0 and 2.0 cc. each are employed for duplicate

analyses. For the best work, the entire oxidation is repeated on another aliquot of serum, so that in all there are four values derived from two oxidations.

Colorimetric procedure. A round-bottomed colorimeter tube calibrated at 5.0 cc. volume is used for the determination. Into this optically calibrated tube is delivered either 3.0 or 2.0 cc. from the solution in the combustion tube. In the latter case additional sodium and acid are supplied to adjust the total ionic strength and acidity to that of the former sample. It is con-

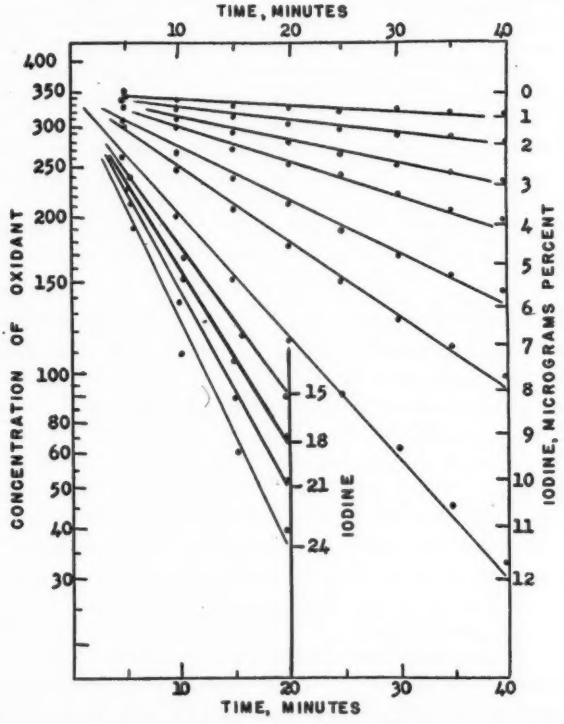

FIG. 1. Nomogram indicating the reduction of 0.001 N ceric ions (oxidant) at 50°C. in 5 cc. volume. The total iodide in the original sample is read on the righthand ordinates, although only one-half of the sample is used in the determination.

venient to prepare a stock solution for this purpose. This is followed by 0.20 cc. of the ceric ammonium sulfate solution (0.050 Normal) and the contents of the tube are mixed well by rocking the tube carefully in a water bath at about 50°C. With a stop watch in readiness, 0.5 cc. of the arsenious acid solution is added. At this instant the stop watch is started at zero, and a few drops of water are added quickly to make the contents of the tube up to the 5.0 cc.-mark. The contents of the tube are mixed quickly by agitating the tube, and an initial reading is made in the Klett-Summerson colorimeter. Corning filter No. 42 is used. The time is noted, and the tube is then placed in a rack in the water bath at 50°C.

The stop watch is kept going and subsequent tubes are treated in the same way, but the respective times of their mixing are noted, so that one watch suffices for a number of determinations. After intervals of ten and thirty-five minutes, each tube is removed seriatim from the water bath, quickly dried, and a reading taken in the colorimeter. The exact time of reading is noted along with the reading itself. This reading is then plotted against its appropriate time on the standard nomogram which has been prepared already as illustrated in Fig. 1.

A line is drawn through the following three points: at the initial time, at the ten-minute reading and at the thirty-five minute reading. Note that these time-intervals need not all be uniform, provided that the corresponding reading is plotted at the appropriate time. These points should fall on a straight line, the slope of which depends upon the iodide present. Accordingly, a straight line is drawn from 350 on the ordinate approximating the slope of these three points. Where it intersects the forty-minute ordinate, the point on the scale at the right of the nomogram (see Fig. 1) will indicate directly the iodine content of the sample in the combustion tube. Ordinarily this sample would be 1.0 cc. of serum, so that the scale reading gives at once the concentration in the blood serum being analyzed.

STANDARD NOMOGRAM

In order to interpret the results of the colorimetric readings, it is necessary to have a standard chart or nomogram (Davis, 1943) from which each reading at a given time can be converted into a corresponding amount of iodide present in the aliquot of material taken for observation. Such a chart is illustrated in Fig. 1. It is prepared on semi-log paper with double-log scales on the ordinate at the left, and with time plotted on the abscissae. The ordinate *at the right* contains a scale which indicates the amount of iodine in the original sample. Because the Klett-Summerson colorimeter dial (Summerson, 1939) bears a logarithmic scale, the ordinate indicates the concentration of the substance, i.e., ceric ion. Of course, other types of colorimeter may be used if due regard is paid to logarithmic function. Since $-dS = Sk \cdot dt$, at any given concentration of iodide the rate of reduction of the colored ion is a linear function when plotted as shown in Fig. 1. It is well to take readings every ten minutes, and to fit the line to the greatest number of points. In any case it should start at 350, and follow the slope indicated by the series of successive readings.

In preparing the nomogram, a series of known samples of iodide are set up in combustion tubes, and these are treated exactly as an oxidized sample would be handled. Appropriate lines are drawn through the readings and a scale constructed to correspond to integral concentrations of iodine. In constructing this scale it may be convenient to transcribe the readings at the right-hand ordinate, i.e., on the forty-minute abscissa, onto a separate chart in which the reading is plotted against iodine. By this method a continuous curve which is nearly linear can be constructed and points transferred back onto the right-hand ordinate of the original nomogram in making the scale.

Alternatively, these points can be transferred to a ruler or a movable scale which may be applied to each new sheet of plotting paper as it is used in routine work. This last device is perhaps the most convenient. An illustrative series of determinations is given in Table 1.

When a poor guess has been made as to the iodine in the sample taken for combustion, certain adjustments may have to be made in analyzing the second aliquot from the combustion tube. If the iodide is very low, it may be desirable to reinforce the tube with 0.02 micrograms of inorganic iodine from a working standard solution. This amount would then be subtracted at the end. If, on the other hand, the combusted sample contains a high amount of iodine, it is possible to take an aliquot less than 2 cc. In this case, however, it is necessary

TABLE 1. ANALYSIS OF KNOWN IODIDE SOLUTIONS

Colorimeter reading at 40 minutes	Iodide in sample μg. $\times 100$	Iodide determined* μg. $\times 100$	Comment
302	Blank	0.3	
302	Blank	0.3	
183	1.3	1.3	Evaporated to dryness
130	2.0	2.1	Evaporated to dryness
128	2.0	2.1	Evaporated to dryness
115	2.7	2.4	Evaporated to dryness
85	3.0	3.0	Evaporated to dryness
54	4.0	4.0	Evaporated to dryness
198	1.3	1.2	Ashed
132	2.0	2.1	Ashed

* The blank is automatically compensated for by the equation or graph, log $350/R = k \cdot I$, where R is the colorimeter reading at 40 minutes and I is the iodide concentration. The constant k is determined empirically for any combination of thermodynamic conditions.

to compute the amounts of soda and of sulfuric acid which thereby are omitted from the final analytical sample, and to add these from a separate source; so that the ultimate solution as made up contains the usual amount of electrolyte and of acid. The final reading is made as before, but it is increased by dividing by the fraction of a cc. taken for the colorimetric determination. For example, if two-tenths of a cc. were taken from the combustion tube, i.e., one-tenth of the usual smaller amount, it would be necessary to supply an additional 99 milligrams of sodium carbonate and 0.70 cc. of 7 normal sulfuric acid; and the answer would be multiplied by thirty to give the iodine in the burned sample.

It is wise to make two determinations of each oxidized sample, and to conduct the combustion in duplicate. If further accuracy is desired, the determination may be followed immediately by a control iodide determination which contains the amount of iodine previously determined as the unknown. If duplicate determinations of such known iodide vary significantly from the results determined in the sample, a proportional correction may be made. Such a procedure would com-

pensate for unexpected deviations in the temperature of the water bath and similar variables.

Accuracy of the method. The over-all accuracy of the method depends upon how many determinations are made. If one oxidizes two samples of serum, and for each sample estimates two aliquots, these four results, when averaged, should have an over-all standard error well under 1.5 micrograms per cent. For working clinical conditions, it is desirable to use one combusted sample, with two aliquot determinations. If these give an average answer which is outside the arbitrary normal limits, a second sample of serum should be combusted. For clinical research and for fundamental problems involving small animals, it is desirable to oxidize two samples of serum.

Determination of iodide in serum. The original supernatant liquor, which is set aside following the coagulation of the protein, is available for determination of *iodide*. The acetic acid should be neutralized with one or two drops of concentrated ammonia (iodine-free). The sample is then treated as if it were a sample of organically bound iodine. If it proves to have a concentration of iodide representing more than 10 micrograms per cent in the original serum, a correction must be applied to the protein-bound iodine, which will be unduly high as analyzed. This correction is due to bound iodide, as described by Bassett, Coons and Salter (1941). If the iodide in the serum is higher than 50 micrograms per cent, it is necessary to dialyse the serum over-night in a small cellophane sac (Riggs, Lavietes and Man, 1942) before proceeding to analyze the organically bound iodine.

IODINE IN SERUM

The following representative values, shown in Table 2, were obtained in various sera. It will be noted that in one hyperthyroid patient, the characteristic effect of thiouracil (Dempsey and Astwood, 1943) had not been attained fully as judged on clinical grounds. Blood from the monkey was obtained from the femoral artery. Blood from the rat was obtained by cardiac puncture.

IODINE IN THYROID OF SMALL ANIMALS

When thyroid iodine, or hydrolytic products thereof, are analyzed, it may be desirable to use the method described elsewhere (Sappington, Halperin and Salter, 1944) because the values will be in the so-called "pharmacological range." If, however, very small animals are used, or if the thyroid is very low in colloid, the present method may be adopted provided the sample is treated first to produce a crude homogenate of the tissue. This may be done, for example, by dissolving in a small amount of 0.02 Normal sodium hydroxide and warming. It may be necessary to triturate the samples with a small glass rod during the process. If it turns out that too much iodine has been taken,

TABLE 2. "HORMONAL" IODINE IN BLOOD SERA

Source of serum	Total iodine	Correction for iodide	Calculated "hormonal" iodine
	μg. %	μg. %	μg. %
Patient L. Hyperthyroidism (San Francisco)	14.8 13.2 Av. 14.0	2.9	11.1
Patient B. Hyperthyroidism (Boston)	11.9 12.9 Av. 12.4	1.9	10.5
Patient McA. Hyperthyroidism; poor response to thiouracil. (New York)	17.8 16.1 Av. 17.0	5.1 4.5 Av. 4.8	12.2
Patient J. Exophthalmic ophthalmoplegia (San Francisco)	7.5 9.8 Av. 8.7	2.4	6.3
Normal woman, P.W. (San Francisco)	8.7 8.7 Av. 8.7	1.9 2.3 Av. 2.1	6.6
Patient R. Formerly hyperthyroidism; good response to thiouracil. (New York)	10.6 9.6 Av. 10.1	5.5 3.0 Av. 4.3	5.8
Patient K. Euthyroid. Rheumatic fever. (New York)	9.0 9.6 Av. 9.3	3.4	5.9
Patient E. Marked myxedema (Boston)	5.7 3.5 Av. 4.6	3.1 3.3 Av. 3.2	1.4
Macacus rhesus (New Haven)	6.6 10.5 8.5 Av. 8.5	3.6	4.9
Albino Rat (New Haven)	7.9 8.3 Av. 8.1	4.2	3.9

the analysis may be salvaged by taking a smaller aliquot and adding compensatory soda and acid as described above. The data in Table 3 will illustrate the determination of iodine in the thyroid of normal adult rats and of rats treated with thiouracil. The results are consistent with those of Astwood and Bissell (1944). The variation in the data is well outside the standard error of the analytical procedure.

TABLE 3. TOTAL IODINE IN THYROIDS OF FEMALE RATS ON STOCK DIET* CONTAINING 1% THIOURACIL

Day of experiment	Stock diet *only*				Stock diet + Thiouracil			
	Wt. of animal (Gms.)	Thyroid (fresh)			Wt. of animal (Gms.)	Thyroid (fresh)		
		Weight mg.	Total iodine micrograms	% iodine		Weight mg.	Total iodine micrograms	% iodine
2						23.4	13.7	0.059
3		11.4	4.3	0.038	220	18.4	12.4	0.067
4						24.2	9.46	0.039
5					274	28.4	12.7	0.045
6	234	14.9	13.5	0.090		19.3	13.2	0.068
7					210	29.4	6.0	0.021
9	320	16.5	17.9	0.108	300	25.7	1.57	0.0061
10					284	38.7	11.0	0.024
11	244	15.0	15.7	0.087				
15					310	78.2	3.28	0.0042
22		15.4	6.4	0.042	235	33.0	2.33	0.0068
27	218	15.2	17.5	0.115	200	30.0	0.8	0.0027
37					248	63.7	3.26	0.0051
43					220	53.2	4.84	0.0091

* The stock diet contained 89 micrograms of iodine per 100 grams.

DISCUSSION

Many problems in iodine metabolism which might be solved with small animals have been delayed for lack of suitable analytical techniques, as pointed out by Salter (1940). It is hoped that the method described here will expedite such problems. By this procedure the conversion of diiodotyrosine into thyroxine may be studied. Likewise, thyroid function may be estimated in the presence of drugs like thiouracil which produce an extraordinary hyperplasia of the gland while the function of the gland declines.

There is good evidence (Salter, Bassett and Sappington, 1941; Riggs, Gildea, Man and Peters, 1941) that the concentration of protein-bound iodine in human serum or plasma is a reliable index of net thyroid function. Determinations of this "hormonal" iodine can be made in untrained animals or subjects, in contradistinction to the determination of basal metabolic rate, even when the serum is transmitted hundreds of miles in appropriate containers. Because four results can be obtained with as little as 2 cc. of serum, the overall accuracy of this procedure should be adequate for many biological and clinical problems. The apparatus required is simple and available already in many laboratories. Several analyses can be made simultaneously.

The rate at which iodide catalyzes the reduction of ceric ions, as shown by Sandell and Kolthoff (1937), is influenced by several factors which must be controlled carefully. Chief among these are 1) temperature, 2) acidity, 3) total ionic strength, and 4) iodide concen-

tration itself. Each experimenter should construct his own nomogram to correspond to the conditions under which he operates. If new complications arise, it will often suffice to compare a control analysis of a working standard solution of potassium iodide which reproduces the innovation. This known control is then read off on the standard nomogram, and if found to be erroneous, the same correction is applied proportionately to the unknown sample. Such a device will take care of a certain number of new experimental problems.

There are four pitfalls which must be avoided. As shown by Sandell and Kolthoff (1937) the catalysis is prevented by the presence of an excess of mercuric ion. The result is explained by the combination of mercury with iodide to form the insoluble mercuric iodide. This complication may be tested for by reinforcing, with a small amount of known iodide, any reaction mixture which fails to show a reasonable reduction of the ceric ion. If an inhibitor is present, the reinforcing iodide will also have little or no influence. Another pitfall is the presence of significant amounts of osmium. This ion catalyzes the reaction markedly. Fortunately such contamination has not been encountered in our work up to the present time. Whenever extraordinarily high values of iodide are encountered, however, this possible complication must be investigated.

Another difficulty is that caustic soda dissolves the Pyrex glass during the baking. The material so released, possibly silica itself, inhibits the catalytic reaction. Unless platinum vessels or alkali-resistant glass tubes are used, sodium carbonate is preferable to sodium hydroxide.

Finally, the surface of the colorimeter tubes should be kept equilibrated with the reaction medium until just before use. Otherwise apparent loss of iodine will occur. This may be done readily by leaving the previously analyzed solution undisturbed until just before the next analysis is performed.

Other micromethods are available, notably those of Christensen (1943) and Talbot, Butler, Saltzman and Rodriguez (1944). The first is a modification of the Groák method, in which the protein is first removed. This procedure involves serious danger of loss of protein-bound iodine. The method is not adequate for the physiological range. The second procedure is a modification of the Riggs-Man method in which the starch-iodide color is measured. It involves a distillation and requires careful control of the varying erythrodextrin pigments produced by different samples of starch. The preliminary separation of the various iodine fractions in biological materials has been reviewed by Wilmanns (1943).

SUMMARY

Organically bound iodine has been measured on 1 cc. samples of human serum and rat serum. Likewise, the thyroids of rats have been

analyzed. The method used involves colorimetric measurements of the rate of reduction of ceric ion as catalyzed by iodide. It is suggested that this method affords a convenient way of measuring net thyroid function directly. It is also possible to investigate the intermediary metabolism of iodine in the thyroid and other tissues of small animals.

This method was tested by Miss Priscilla Wheeler, visiting technician from the Department of Medicine, University of California, who contributed valuable suggestions as to routine procedure.

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NOTES AND COMMENTS

ALLOXANTIN AS A DIABETOGENIC AGENT IN RABBITS

DUNN and co-workers (1943) found that alloxan produced selective alterations in the islets of Langerhans and suggested in their first publication the possibility of obtaining by this agent a permanent experimental diabetes. Before this Jacobs had found that the intravenous injection of alloxan provoked in rabbits after a short period of hyperglycemia a distinct hypoglycemia, even leading to the characteristic hypoglycemic shock. He could not get the same effect with other compounds of the alloxan group, including alloxantin.

In the course of our investigations on secondary factors intervening in alloxan-diabetes we tried to find out, if other compounds of the alloxan group have the same diabetogenic effect. Among these we tested alloxantin, chemically a near relative of alloxan, which can be considered the product of the oxidation of uric acid or of the reduction of alloxan. These three substances are highly specific receptors of hydrogen ions and consequently strong oxidants.

We prepared the alloxantin for our experimental work by oxidation of uric acid, following the method described in Gillman (1943). The alloxantin obtained was a white crystalline powder, which turned red when exposed to air, and had to be kept in a vacuum. It decomposed at 246–248°C., its solubility in cold water very low and it was much more soluble in hot water. By this property it can be distinguished from alloxan, which dissolves fairly well in cold water and also by a characteristic reaction with $Ba(OH)_2$. Solutions of alloxan give a white precipitate with $Ba(OH)_2$ and alloxantin a violet one.

The low solubility of alloxantin obliged us to use hot solutions, of about 50°C., for the intravenous injections in rabbits. We injected two different concentrations, one of 1% and the other of 2.5%, and it was generally given in doses from 70–230 mg. per Kg. of body weight. Both solutions caused acute pain and violent general reactions while the injected veins became thrombosed.

It was remarkable that the smaller concentration was more toxic than the higher. In 1% solution, doses of only 70–80 mg. per kg. of body weight killed the animals, producing convulsions, protruding eyeballs and extreme dilatation of the pupils. Only one of four animals injected in this way survived, although with symptoms of acute intoxication.

The remaining 12 animals (4 ♂, 8 ♀) were treated with solutions of 2.5% in doses between 100–230 mg. per kg. Only one female died during the injection in the same violent way as the animals treated with the 1% solution. Another female died the next morning. No animal showed the convulsion of hypoglycemic shock, so frequently observed when alloxan is used. Two of

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the eight females had real diabetes with glycosuria and hyperglycemia. Of the four males one got severe diabetes after a reinjection and another one suffered, also after reinjection, rather late and temporary glycosuria. The two remaining ones had only transient glycosuria.

The histological examination revealed the typical lesions of the Langerhans islets, also observed with alloxan. These included cellular degeneration and necrosis, marked diminution of the beta cells and in cases of longer duration diminishing amounts of islet tissue. Very instructive was the case of one

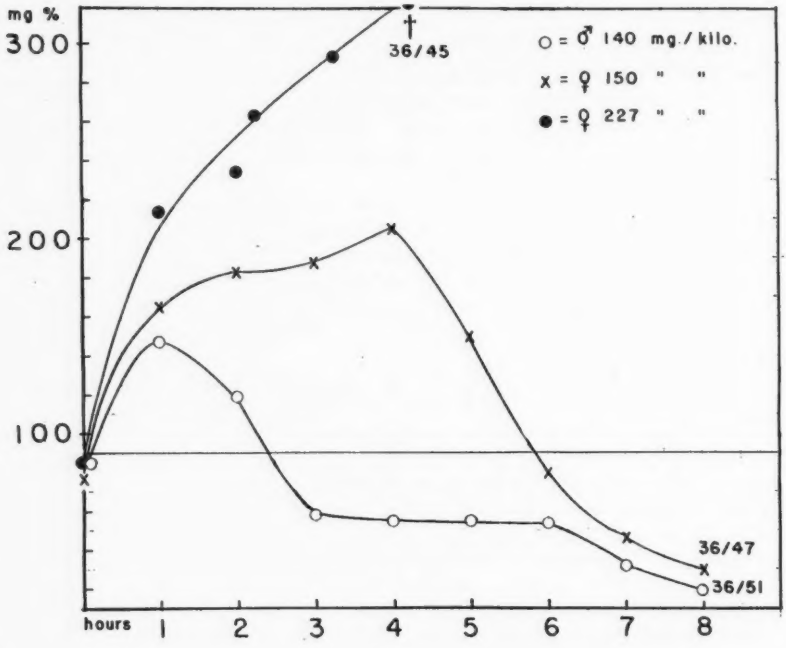

FIG. 1

animal in which the number of islets was markedly reduced and the remaining ones were small and largely necrosed. The former alteration corresponds to the first, the latter to the second injection. This animal got diabetes only after the second injection. Animal 36/45 died four hours after the injection, with severe circulatory disturbances and hyperglycemia. The histological examination revealed only diminution of the beta cells in all the islet of Langerhans, but no symptoms of any degeneration or necrosis.

In several animals we found, besides these specific alterations of the islets, lesions in the pancreatic parenchyma and also in the convoluted tubules of the kidneys similar to those seen in alloxan intoxication.

Although Jacobs could not demonstrate changes in the blood sugar with alloxantin, we repeated his procedure with 2.5% solutions of alloxantin, checking blood sugar hourly, during a period of eight hours after the injec-

tion. The curves show the blood sugar reaction in three animals. Animal No. 36/45 died four hours afterwards with symptoms of pulmonary edema. We can see in the curves that their behaviour is similar to those produced by alloxan, only that perhaps it can be said, that the hyperglycemic phase is somewhat prolonged. The two animals with a very low blood sugar gave no signs of hypoglycemic shock, and the following day they were perfectly well. (Fig. 1.)

It would appear that alloxantin may produce experimental diabetes, but there are apparently certain differences from the effects of alloxan. First of all there is the paradoxical effect that the lower concentrations, even administrated in small doses, are more toxic than the larger doses in higher concentration. This may be explained by the supposition that the alloxantin, like alloxan is mainly a capillary poison (Labes and Friedberger). If such a compound is administered in higher dilution, its diffusibility will be increased, especially in cases of relatively insoluble substances. Consequently it could paralyse more rapidly the capillary system. Higher concentrations with tendency to crystallise, may act more slowly, developing rather the symptoms of subacute intoxication. The second fact is that none of the animals treated in our experiments with alloxantin developed hypoglycemic shock, although we could demonstrate that the blood sugar figures reached a very low level.

Investigations by Hughes, Ware and Young, and Gomori and Goldner, suggest that the hyper- and hypoglycemia produced by alloxan poisoning can be explained by the effect of adrenalin, followed by insulin, when the latter is freed abruptly by necrosis of the islet cells. It seems that the hyperglycemic phase is more prolonged with alloxantin and it at least in one animal, No. 36/45, that necrosis and degeneration of the islets do not develop as rapidly as in alloxan poisoning. In its diabetogenic action alloxantin is not different from alloxan.

Summary. Intravenous injection of alloxantin produces diabetes mellitus in rabbits. Histological examinations reveal a selective affinity of alloxantin for the cells of the islets of Langerhans. The glycemic curve in alloxantin poisoning shows a prolonged phase of hyperglycemia, which lasts from 2 to 5 hours followed by a very marked phase of hypoglycemia. 1% solutions of alloxantin are more toxic than 2.5% solutions.

Hypoglycemic shock was not observed with alloxantin.

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NEW BOOKS

PROBLEMS OF HUMAN FERTILITY. Edited by Earl T. Engle. Published for the National Committee on Maternal Health by the Banta Publishing Co., Menasha, Wisconsin, 182 pp. 1944.

For the last four years, the National Committee on Maternal Health has held annual round table discussions at which various aspects of the Biology of Human Fertility have been discussed. The success of these informal conferences has been attested by a noteworthy demand for distribution of the Proceedings. The present volume contains the reports and discussions presented at the last meeting where the topics discussed were *Criteria of Ovulation, Some Biological Observations on the Lower Generative Tract,* and *The Placenta as a Barrier.*

Of the various methods sometimes employed for the determination of ovulation, four were selected for discussion. The validity of endocrine procedures, especially the excretion of pregnandiol, was discussed by Dr. E. L. Sevringhaus. Embryological criteria were considered by Dr. John Rock in a study of endometrial and ovarian histology as correlated with the occurrence of tubal eggs at known times of the cycle. Dr. W. W. Greulich presented data on the variations in body temperature during the cycle as they relate to the histology of the corpus luteum. Finally, a comprehensive account of the studies on ovulation potentials carried out in Burr's laboratory was submitted by Dr. J. L. Boling. In general, the internal consistency of the data obtained by these various methods affords considerable assurance in our statistical ability to predict the time of ovulation, but the curse of variability still defeats attempts to detect ovulation accurately in all instances.

The second section deals with physico-chemical studies of the milieu in which spermatozoa exist during their sojourn in the female reproductive system. Drs. J. K. Lamar, Boris Rubenstein and A. E. Rakoff considered various aspects of the chemical and physical compositions of the cervical and vaginal secretions as related to the transport and viability of spermatozoa. Although considerable progress appears to have been made, the difficulty imposed by the small quantities of material which can be secured seems to have prevented the collection of thoroughly satisfactory data. The reviewer is tempted to observe that some of the recent microchemical procedures might well find application in this difficult field.

The final section of the conference dealt with the problems arising from the mechanical separation of the fetal and maternal blood streams. Dr. George B. Wislocki presented an account of the cytology of the trophoblast, Dr. F. F. Snyder gave an account of studies showing how placental transmission varies during pregnancy, and the transmission of radioactive elements and of native proteins was recounted by Drs. Alfred Gellhorn and Bret Ratner. These studies mark a new era in placental physiology. It is of course

true that sporadic efforts to consider the placenta in physico-chemical terms have been attempted before. However a concerted attack of the problem, using the varied armamentaria of the physicist, chemist and biologist, is new and is providing exciting data.

A final word on the style of the volume is perhaps in order. Stenographic copies of the verbal reports and discussions were prepared and used as rough drafts of the various manuscripts. While this device unquestionably makes for a fresh and unhackneyed presentation, it is nevertheless unfortunate that most of us write a somewhat better brand of English than we speak. Consequently, while some of the authors apparently have revised their manuscripts to conform to standards of written material, others have allowed awkward phrases and faulty organization to remain in the final copy. Since the nuances conveyed by tone and expression in the verbal exposition have found no counterpart on the printed page, they are unhappily lost to the reader.

EDWARD W. DEMPSEY

THE ADRENAL GLANDS IN HEALTH AND DISEASE. By Max A. Goldzieher, M.D. Endocrinologist, St. Clare's Hospital, New York City. Fabrikoid, $18.00. Pp. 727 with 81 illustrations. Philadelphia: F. A. Davis Co., 1944.

Endocrinology and particularly adrenal physiology have advanced tremendously since the publication of Dr. Goldzieher's first book on "The Adrenals" in 1911. The present volume has brought up to date a comprehensive literature on the adrenal which has accumulated since Grollman's review eight years ago. The book is divided into four parts under which are discussed 1. Morphology, 2. Physiology, 3. Diseases of the Adrenals, and 4. Pathophysiology. Little of note has been added in recent years to our knowledge of "morphology" of the adrenals, whereas the chapters devoted to discussion of "physiology" and "diseases of the adrenals" are replete with new references. The material is presented in excellent form. The chapters devoted to "pathophysiology" should prove stimulating to investigators in the field as the subject matter discussed is particularly controversial. The book throughout is exceptionally well documented, and the author should be complimented on the nice balance which he has attained between discussion of structure, function and clinical significance of both medulla and cortex. For the first time we now have available a single reference in which the clinical discussion of the treatment of diseases of the adrenal glands approaches the scientific level of the discussion of the physiological action of epinephrine. To all interested in endocrinology and particularly to those interested in adrenal physiology this book is highly recommended.

GEORGE W. THORN, M.D.

ANNUAL REVIEW OF PHYSIOLOGY. Vol. 6. James Murray Luck, Editor. $5.00. vii+630 pp. American Physiological Society and Annual Reviews, Inc. Stanford Univ. 1944.

The principle of Annual Reviews is decidedly valuable to persons engaged in research and teaching in the varied fields included under the term "physiology." In this volume we find interesting accounts of subjects as far

apart as "cocarcinogenesis" and "psychosurgery." Even if we look upon the chapters as mere compendia of recent work, as the poorer ones are, we find them superior to lists of articles or abstracts in being orderly. Important in this respect is the good subject index. But such chapters cannot be written without at least a minimum of critique. In the best chapters this is an outstanding virtue.

Critique implies that the author has an opinion. A most important function of these reviews is the expression of opinion by workers active in the fields they are covering. The best chapters rank high in this respect. The expressed opinions, concepts, and attitudes help to formulate problems and orient the direction of research.

An occasional pithy chapter or paragraph enlivens this otherwise earnest book.

G. H. Acheson

ASSOCIATION NOTICE

MINUTES OF THE TWENTY-SEVENTH ANNUAL MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

THE TWENTY-SEVENTH Annual Meeting of the Association for the study of Internal Secretions was held Monday and Tuesday, June 12 and 13, 1944, at the Stevens Hotel, Chicago.

During the scientific sessions a program (published elsewhere)* was presented:

MINUTES OF THE MEETINGS OF THE COUNCIL

FIRST MEETING

The Council met at 7:00 P.M., June 11, 1944, in Private Dining Room No. 8 of the Stevens Hotel, Chicago.

Members present were: Drs. Shelton, Moore, Engle, Hoskins, Kenyon, Rynearson, Sevringhaus, and Turner. Absent were: Drs. Burch, Goldzieher, Thorn, Smith, and Allen (deceased). Members of the Publication Board present were: Drs. Hamblen, Thompson, MacKay, and Managing Editor, Dr. Astwood, and Assistant Managing Editor, Dr. Dempsey; Dr. Nelson of the Committee on Awards, and Mr. Charles C Thomas, Publisher.

President Shelton presided.

In the absence of Dr. Perry McCullagh, Chairman of the Nominating Committee, the Secretary, Dr. Turner, reported the following nominations for 1944–45:

President-Elect	Fuller Albright
Vice-President	Willard O. Thompson
Secretary-Treasurer	Henry H. Turner

Members of the Council for Three Years (1944–47):

Robert Gaunt	E. Kost Shelton
Edwin B. Astwood	Jean Paul Pratt (2 years) (To fill unexpired term of Edgar Allen)

Members of the Publication Board for Three Years:

Earl T. Engle	Elmer L. Sevringhaus

The Secretary-Treasurer's report was read by Dr. Turner and accepted by the Council.

Dr. Rynearson, Chairman of the Committee on Awards, which consisted of Drs. Hamblen, Thompson, Rynearson, Nelson, and Smith, reported that Dr. E. A. Doisy of St. Louis had been chosen as recipient of the 1944 Squibb Award, and Dr. E. B. Astwood of Boston as recipient of the 1944 Ciba Award. It was further stated that both awards will be available next year.

* *Endocrinology* 35: 199, 1944.

Upon motion by Dr. Sevringhaus, seconded by Dr. Engle, the Editor's report was accepted. The report, given by Dr. Hoskins, was as follows:

"This report will comprise two sections, namely that for both Journals for the year 1942 by Dr. R. G. Hoskins and that for 1943 by Drs. E. B. Astwood, E. W. Dempsey, and R. G. Hoskins.

"Early in the summer of 1942, Dr. Milton O. Lee resigned the Editorship of the two Journals preparatory to entering the military service, which he did in the early autumn. He was succeeded by Dr. Hoskins, who served as Managing Editor for both Journals, having the assistance of Dr. Rucker Cleveland. Beginning with the February issue in 1943, the Editorship of ENDOCRINOLOGY was taken over by Dr. Astwood as Managing Editor, with Dr. Dempsey as Associate Managing Editor. The Editorial Committee who had previously cooperated with Dr. Lee for THE JOURNAL OF CLINICAL ENDOCRINOLOGY continued to serve under the new management. During 1943, Dr. N. B. Talbot joined the Editorial Committee. Upon assumption of the Managing Editorship, Drs. Astwood and Dempsey secured the services of an Editorial Board for ENDOCRINOLOGY, consisting of Drs. C. H. Best, J. S. L. Browne, E. T. Engle, C. G. Hartman, F. L. Hisaw, E. C. Kendall, F. C. Koch, C. N. H. Long, and H. B. vanDyke. Dr. Cleveland continued as assistant to the Editors until February, 1944, when she resigned to accept employment elsewhere.

"With Dr. Cleveland's departure, the joint Editorial Office at the Harvard Medical School was discontinued, that for ENDOCRINOLOGY being transferred to a room in the Department of Anatomy. Mrs. Mildred E. Rider, who had previously served as secretary for the joint office, transferred to the ENDOCRINOLOGY Office at this time. Mrs. Barbara B. Banay assumed the position of Assistant to the Editor of THE JOURNAL OF CLINICAL ENDOCRINOLOGY, serving on a part-time basis. The decentralization of the two offices was completed, except the Editorial Work Fund continued to be utilized in common, it remaining in the custody of Dr. Hoskins.

"Occasion was taken at the time of Dr. Cleveland's departure to re-study the activities of the Editorial Office with a view of eliminating certain procedures which had to do with matters of business rather than editorial function. A conference was held to this end, with Mr. Charles C Thomas, our Business Manager, Mr. Mark Bradford, who represented the George Banta Publishing Company, Drs. Astwood, Dempsey, and Hoskins. A few and relatively minor changes of procedure, only, appeared to be advisable.

1. The entire matter of negotiating for, ordering and collecting for reprints was turned over to Mr. Thomas.
2. All other business correspondence was turned over to Mr. Thomas.
3. Checking monthly printing statements for accuracy and certifying these to the Treasurer for payment was transferred to Mr. Thomas.
4. The Banta Company undertook the responsibility for topographical treatment of titles, sub-titles, etc., and for determining the order of appearance of articles, except as specified in special cases by the Editors (this is to facilitate adjustment of text to signatures with a view to subsequent reprinting).

"That arrangement left to the Editorial Office these functions:

1. Consideration of manuscripts as to suitability for publication and arranging with authors for revisions of manuscripts.
2. Preparation of manuscripts for printing (editing proper), indicating subdivisions that should appear in reduced type size.
3. Editing galley and page proofs, incorporating suitable changes desired by authors.

"These changes, it appeared, would add somewhat, but not greatly, to the work of the Business Manager and the manufacturer. It was agreed that after a trial period to determine the magnitude of the additions, recommendations would be made to the Council for readjustment of reimbursements to these two agencies.

"As was foreseen, the existing emergency condition added significantly to the difficulties of conducting the Journals. In particular, the inflow of manuscripts was materially reduced. In case of the CLINICAL JOURNAL, an attempt was made to remedy the difficulty by solicitation of special articles, mostly reviews, from various authors. The Editor wishes especially to thank those who were able to respond favorably. Nevertheless, succeeeding issues became increasingly attenuated in size and late in appearance so that the January, 1944, issue did not reach the subscribers until April, 1944. In the meanwhile, material for three further issues had been received and transmitted to the printers so that early in April three issues were simultaneously in press. Some of the delay in publication was thus made up, but paucity of manuscripts following that period in part led to cancelling of the gain.

"The following are: (a) Report of Dr. Hoskins for ENDOCRINOLOGY and THE JOURNAL OF CLINICAL ENDOCRINOLOGY for 1942, (b) Report of Drs. Astwood and Dempsey for ENDOCRINOLOGY for 1943, and (c) Report of Dr. Hoskins for THE JOURNAL OF CLINICAL ENDOCRINOLOGY for 1943.

"The financial reports for 1942 are based on records kept in the Editorial Office; those for 1943 are taken from the Auditor's Reports. Prior to 1943, the accounts were kept on a cash basis, that is to say, the total costs and total income of each volume of the Journals were determined as completed transactions. Beginning with 1943, the accrual basis was adopted, that is to say, the year of business activities for each Journal is reported as such without regard to carry-over items of income and expenditure beyond January 1 into the succeeding year.

"Respectfully submitted,

6/10/44 (signed)

R. G. Hoskins, Managing Editor."

ENDOCRINOLOGY
1942

Material Published

Total Pages of Text 1693

257 Original Articles

Circulation

Subscriptions (figure as of August, 1942)	1325
(Of these, 270 were for foreign subscribers)	

Financial Statement

Income	$24,798.74
Disbursements	21,988.35[1]
Net Profit	$ 2,810.39

JOURNAL OF CLINICAL ENDOCRINOLOGY

1942

Material Published

Total Pages of Text	776

Circulation

Subscriptions (figure as of August, 1942)	1204
(Of these, 184 were for foreign subscribers)	

Financial Statement

Income	$18,283.70
Disbursements	15,484.28[2]
Net Profit	$ 2,799.42

ENDOCRINOLOGY

1943

In 1943, 176 original articles were submitted for publication. Of these, 101 were accepted, 12 were accepted only after extensive revision, and 63 were rejected. These figures show a continuous falling off of material submitted, since in 1942, 269 articles were received, of which 23 were rejected.

Material Published

Total Pages of Text	908

Circulation

Subscriptions (figure as of August, 1943)	1294

Financial Statement (Cash Basis)

Income	$22,643.80
Disbursements	15,713.29
Net Profit	$ 6,930.51

THE JOURNAL OF CLINICAL ENDOCRINOLOGY

1943

Material Published

Total Pages of Text	743

[1] As of July 1, 1942, there was a 6% increase in labor costs for manufacture.

[2] There was a 10% increase in the cost of manufacture as of July 1, 1942, 6% being for labor increases, and 4% for adjustment in the original estimates of manufacture.

Circulation

Subscriptions (figure as of August, 1943)..... 1308
(Of these, 230 were foreign subscribers)

Financial Statement (1/1/43–12/31/43)

Figures taken from the report of H. E. Cole Company for the Association and represent a change in the accounting system used for previous years. Amounts given represent completed transactions for the calendar year.

Income	$18,971.86
Disbursements	12,617.86
Net Profit	$ 6,354.00

Respectfully submitted,
(signed)
R. G. Hoskins, Managing Editor
(signed)
E. B. Astwood, Managing Editor
(signed)
E. W. Dempsey, Asst. Managing Editor

(First Council Meeting, Contd.)

Motion was made by Dr. Sevringhaus, seconded by Dr. Kenyon, that the Secretary-Treasurer be authorized to purchase $10,000 in War Savings Bonds. Carried.

Dr. Sevringhaus, Chairman of the Committee on Assays and Standards, gave the following report:

"The Committee on Endocrine Assays has not had formal meetings since the last meeting of the Association, but the items have been discussed informally on a number of occasions. Under date of October 8, 1943, the Chairman was notified that he had been appointed a member of the U.S.P. Endocrine Products Advisory Board, of which Dr. Charles L. Brown is Chairman and the other members are Dr. Willard M. Allen and Dr. George W. Thorn. This group met in Washington on January 9, 1944, and had associated in the meeting representatives for the different endocrine products, including almost identically the individuals who had already been appointed for the Assay Committee of the Association for the Study of Internal Secretions. An entire day was spent in the discussion, and it was felt that much progress was made toward an understanding and agreement for the inclusion in the forthcoming revision of the United States Pharmacopeia of a number of endocrine products, together with statements about their purity and methods of assay. Among other things, a method is being worked out by which the assay techniques will be published and then commercial firms may be invited to try these methods and report their results by code number so that adequacy, accuracy, etc., can be evaluated by the Pharmacopeia Endocrine Products Advisory Board. This can be done at a minimum of expense. It may become necessary at a later time to ask for some contributions to a pooled fund for part of this work. For the present, it is, therefore, proposed that the Assay Committee of the Association not undertake any definite

program, since the Pharmacopeia activity may be adequate to cover the immediate needs. Your Committee will continue to keep the situation in mind and to report as necessary.

"Respectfully submitted,
(Signed)
Elmer L. Sevringhaus, Chairman."

Due to some changes in operations, certain revisions of the By-Laws were deemed imperative, and upon motion by Dr. Hoskins, seconded by Dr. Sevringhaus, it was voted to make the following changes:

1. Abolishment of the Publication Board and appointment of an Editorial Committee by deleting Section 2 of Article VII.
2. That Section 2 of Article V be amended by omission of the phrase "and on the Publication Board." This is necessitated by deletion of Section 2, Article VII.
3. That Section 4, Article II, be amended by omission of the phrase "by the Secretary-Treasurer."
4. That Section 1, Article VI, be amended by omission of the phrase "plus an annual membership assessment."

Motion by Dr. Turner, seconded by Dr. MacKay, that the revised By-Laws be printed in ENDOCRINOLOGY and copies sent to each member. Carried.

In view of the above, it was moved by Dr. Sevringhaus, seconded by Dr. Rynearson, and carried, that an Editorial Committee, consisting of three members of the Council, be appointed by the President, subject to ratification of the Council. President Shelton appointed Drs. Engle, Thompson, and Sevringhaus, with Dr. Engle as Chairman of the Committee. These appointments were approved upon motion by Dr. Hoskins, seconded by Dr. Turner.

After a rather lengthy discussion by members of the Council, Publication Board, and some remarks by the Publisher, Mr. Thomas, it was moved by Dr. Sevringhaus and seconded by Dr. Engle, and carried, that the Publishers and Business Manager should receive fair remuneration for their work and that their contracts should be renegotiated. Details should be worked out between the Publisher, Mr. Thomas, and Dr. Turner representing the Association, and submitted to the Council for ratification.

Motion by Dr. Hoskins, and seconded by Dr. Kenyon, that the same firm of auditors should audit all association accounts, i.e., those of Mr. Thomas' office and those of the office of the Secretary-Treasurer. Carried.

Motion by Dr. Shelton, seconded by Dr. Engle, that the Roster be published in ENDOCRINOLOGY, with offsets to each member of the Association. Carried.

The Secretary read a letter from Dr. James K. Donahue, in which it was suggested that the name of the Association was entirely too long and should be shortened. Members of the Council were quite in sympathy with this. It was moved by Dr. Moore, and seconded by Dr. Engle, that some suggestions be made for a shorter name and that the Secretary contact the Corporation

Service Company, of Delaware, who act as the Association's agent, with regard to the legal aspects involved in changing the name of the Association. Carried.

The Secretary also read a letter from Dr. Victor Johnson, Secretary of the Council on Medical Education and Hospitals of the American Medical Association, with regard to postgraduate programs which the Association might be giving or contemplating. It was the consensus that the Association might well sponsor such programs in endocrinology. However, no action was taken.

The Secretary read a letter from Dr. Rita S. Finkler regarding organization of a Board of Endocrinology. This was thought not feasible at the present time.

The matter of discrepancy in dues was discussed at great length, At the Association's annual business meeting in Atlantic City, May 3, 1941, it was "moved by Dr. P. J. Daughenbaugh and seconded by Dr. Murray Gordon, that the dues should be set at $10.00 per year and that this should include the choice of either ENDOCRINOLOGY or THE JOURNAL OF CLINICAL ENDOCRINOLOGY, and that the other journal may be subscribed for at the regular subscription rate. Carried." This has seemed rather unfair, in that the members who subscribe only to THE JOURNAL OF CLINICAL ENDOCRINOLOGY (subscription to non-members $6.50) are paying dues or a difference of $3.50, while those who elect ENDOCRINOLOGY (non-member subscription price $10.50) are saved 50 cents. Obviously the member who subscribed to THE JOURNAL OF CLINICAL ENDOCRINOLOGY is penalized $3.50. It is true the Publication Board or Publisher elected a combination offer of both Journals of $15.00 to members only. However, this does not correct the discrepancy. It was suggested that Mr. Thomas and Dr. Turner work out a more equable schedule.

The merits of the Association's publishing certain worthy monographs were discussed, and it was the opinion of the Council that this should be left to the Editorial Committee for consideration and presentation of definite ideas to the Council at some later date. The meeting adjourned.

SECOND MEETING

The Council met June 12, 7:30 A.M., in Private Dining Room No. 6 of the Stevens Hotel, Chicago.

Members present were: Drs. Engle, Hoskins, Kenyon, Sevringhaus, Rynearson, Moore, and Turner. Dr. F. M. Pottinger, one of the founders of our Association, and who served it so faithfully as Secretary-Treasurer for eighteen years and more recently as President, was a welcome guest.

President Shelton presided.

There was much discussion with regard to the editorship of the Journals, inasmuch as Dr. Astwood wished to resign. The Editorial Committee were asked to submit their views at a later date.

The following is report of the Committee, which was forwarded by mail to members of the Council and ratified on August 4, 1944:

"We recommend that Dr. Edward W. Dempsey, Assistant Managing Editor of ENDOCRINOLOGY, be appointed Managing Editor of ENDOCRINOLOGY for a period of three years. This appointment will become effective, on ratification by the Council, with Volume 36, Number 1, January, 1945.

E. L. Sevringhaus
W. O. Thompson
E. T. Engle, Chairman"

Upon motion by Dr. Rynearson, seconded by Dr. Hoskins, seventy-seven applicants were admitted to membership. The names of these applicants will appear in the forthcoming roster.

Dr. Moore announced the following appointments:

Committee on Awards

P. E. Smith, Chairman	(term expires 1948)	
E. H. Rynearson	(term expires 1946)	
W. O. Nelson	(term expires 1947)	
W. O. Thompson	(term expires 1945)	
Carl Hartman	(term expires 1949)	(replacing E. C. Hamblen, whose term expired)

Nominating Committee for 1945

Fred Koch, Chairman
George W. Thorn
Willard M. Allen

The meeting adjourned.

THIRD MEETING

The Council met at 12 Noon, June 12, in the South Ballroom of the Stevens Hotel, Chicago. Those present were Drs. Shelton, Engle, Hoskins, Kenyon, Sevringhaus, Goldzieher, and Turner.

It was moved by Dr. Rynearson, and seconded by Dr. Sevringhaus, that we meet with the American Medical Association next year. Carried.

The Council voted its sincere thanks to Dr. and Mrs. W. O. Thompson for the splendid manner in which they took care of the arrangements for this meeting. A vote of thanks was also extended to the Hotel management for their accommodations and the successful conduct of this meeting.

Dr. Turner reported that he and Mr. Thomas had attempted to arrive at some equable fees for the Journals, but were unable to do so. Therefore, upon motion by Dr. Turner, seconded by Dr. Engle, the dues were set at $10.00 for the ensuing year to include THE JOURNAL OF CLINICAL ENDOCRINOLOGY OR ENDOCRINOLOGY.

On motion, the meeting adjourned.

E. KOST SHELTON, *President*
HENRY H. TURNER, *Secretary-Treasurer*

MINUTES OF THE ANNUAL BUSINESS MEETING

The Twenty-Seventh Annual Business Meeting of The Association for the Study of Internal Secretions was called to order by President Shelton at 5 o'clock the afternoon of June 13, in the North Ballroom of the Stevens Hotel, Chicago.

President Shelton presided.

The sealed ballots were opened and recorded by the tellers, Drs. Dunn, MacKay, and F. M. Pottenger, Jr.

Minutes of the previous meeting were read and approved.

The Secretary-Treasurer's report was read and approved.

The changes in the By-Laws (see First Council Meeting) which were approved by the Council, on motion by Dr. Turner, seconded by Dr. MacKay, were read and approved.

The dues were set at $10.00.

Upon motion by Dr. Turner, seconded by Dr. Thompson, it was voted that the next annual meeting be held in connection with the American Medical Association as it was this year. Carried.

Dr. Leona M. Bayer suggested that perhaps the Society in the not too distant future might find it expedient to appoint a woman on some of its committees. This suggestion was received with applause.

The tellers gave their report to the President, who announced the election of the following officers and members of the Council:

President-Elect	Fuller Albright
Vice-President	Willard O. Thompson
Secretary-Treasurer	Henry H. Turner

Members of the Council for Three Years (1944–47):

Robert Gaunt	E. Kost Shelton
Edwin B. Astwood	Jean Paul Pratt (2 years)
	(To fill unexpired term of Edgar Allen)

(Replacing P. E. Smith, M. A. Goldzieher, and George W. Thorn.)

Dr. Shelton turned over the presiding to Dr. Moore, and after a few pertinent remarks from him, the meeting adjourned.

E. Kost Shelton, *President*
Henry H. Turner, *Secretary-Treasurer*

REPORT OF THE SECRETARY-TREASURER

1944 MEETING

This report covers the years 1942 and 1943, with an addendum for the first five months of 1944.

The last publication of the Roster (Endocrinology, May, 1942) showed the membership to be 760 as of April 1, 1942. During the period April to December, 1942, 81 new members were admitted and 12 requested cancellation of membership, resulting in 829 members as of December 31, 1942. Of this number, 99 had failed to remit their current dues. Fourteen new mem-

bers were admitted in 1943, increasing the membership to 843, of which 235 were in arrears with their dues and 14 had requested suspension of dues for the duration of the Emergency. Sixteen new members have been added in 1944, making the total 859, of which 272 are delinquent and 21 have requested suspension of dues. We have 22 applications to be considered at this meeting.

At the request of the Office of Emergency Management of the United States, the President, Dr. E. Kost Shelton, polled the Council in November, 1942, for permission officially to cancel the 1943 meeting; this permission was granted by the Council. It was also the consensus of the Council that the present officers be continued until the next annual meeting or until such time as the Council deemed it advisable to call another election by ballot.

In January of this year, the President sent questionnaires to each member of the Council, and it was agreed to have a meeting in 1944. Because of the central location of Chicago and the fact that the American Medical Association is meeting here, this was considered the most desirable place.

A special Committee of Officers appointed by the Council at the 1942 meeting in Atlantic City met October 6, 1942, at the Palmer House in Chicago. Those present were Drs. Roy G. Hoskins, Carl Moore, Henry H. Turner, Mr. Charles C Thomas, and the President was represented by his secretary, Miss Kathryn Broderick, who compiled the following summary of the meeting:

1. "It was decided that the Publication Board, as an elective body, should be abolished by deleting Section 2, Article VII of the By-Laws. With this deletion, the Committee has ample power under Section 3, Article IV to suggest to the Council that they appoint a Committee on Publications.

"That an Executive Committee should be appointed by the Council to act in place of the Council, to have general charge of all publication matters, and to replace the present Publication Board. The Council is finally responsible anyway, in that the Publication Board has to report to the Council and have its activities approved by the Council.

2. "That Dr. Henry H. Turner and Mr. Charles C Thomas determine the details of handling the financial matters of the Association, in regard to collection of monies and disbursement of same.

"That Dr. Turner and Mr. Thomas submit to the Council recommendations for a new contract with the Publisher. Dr. Hoskins, Managing Editor, wishes to have no official part in this, since his position involving the details and responsibilities of both Journals should be kept apart from the financial side.

3. "That one auditing firm should be engaged (preferably one having offices in key centers throughout the United States) to audit all books of the Association, and that all books be kept on the same basis, namely, either a cash basis or an accrual basis.

4. "That the Association for the Study of Internal Secretions owns both Journals and that the idea of segregating the Journals from the Association be ruled out.

5. "That the President and the Secretary are responsible for the program of the annual meeting and that they constitute the Program Committee.

6. "That the abstracts of papers submitted to the Program Committee to be read before the annual meeting or to be read by title should be published in the program of the annual meeting and not in either of the Journals. (Dr. Hoskins explained in detail his reasons for this recommendation, and there was general discussion on this point.) It was pointed out that all papers which are desirable for publication will be available to the Journals, and it was Dr. Hoskins' contention that because a paper is submitted, it does not mean that it is deserving of publication or reference in medical literature.

7. "That the Association's records should show a true list of all members and subscribers.

8. "That the list of uncollectable accounts receivable, amounting to $250.18, as shown in report submitted by Mr. P. C. Mason, be charged off. The list included one item of approximately $21.00 which it was unanimously agreed should be refunded to Mr. Mason.

"Regarding discussion of approach to the pharmaceutical houses for advertising in ENDOCRINOLOGY and THE JOURNAL OF CLINICAL ENDOCRINOLOGY:

1. "That it has been the policy of the Association and its Journals for many years to promote and uphold high standards in endocrinology. This policy is definitely to the advantage of the ethical firms in this group in meeting competition with the less particular.

2. "That the Association and its Journals have as a major function the promotion and interest of endocrinology, which in the final analysis keeps endocrine medications before the public.

3. "In this period of war crisis our Journals, like all other medical journals, are confronted with a different set of budgetary circumstances and need the support of advertisers more at this juncture than any other."

In compliance with the request of the special Committee of Officers appointed by the Council at the last annual meeting, Wolf and Company were selected to audit the books of the Publisher and of the Secretary-Treasurer. It will be recalled perhaps that in the past there have been some minor discrepancies in these two reports, occasioned by different methods of bookkeeping and by different auditing procedures. The Secretary-Treasurer is pleased to report that the 1942 audits of the two offices are in accord. Because of the expense involved, and without further authorization by the Council, Wolf and Company have not been asked to audit the books of the Secretary-Treasurer for 1943. Cost of the Wolf and Company audit in 1942 totaled $344.00 ($216.50 for auditing the Publisher's accounts, and $127.50 for auditing the Secretary-Treasurer's books).

The 1943 audit by H. E. Cole Company reflects a substantial gain in net worth over 1942.

Total income for 1943 was $1,014.40 less than for the year 1942, and while the income from ENDOCRINOLOGY was $1,964.43 less than in 1942, the income from THE JOURNAL OF CLINICAL ENDOCRINOLOGY was $890.09 greater than the previous year. Total expense of ENDOCRINOLOGY for 1943 was $5,483.93 less than in 1942, and JCE was $2,002.68 less. Total net income for 1943 was $9,385.84 over 1942.

This striking gain is due, of course, to the decrease in size of the two Journals, necessitated by the lack of acceptable material for publication.

The President has requested that all members keep in close touch with the Editors regarding material for the Journals. You are aware of the dearth of manuscripts, which has been occasioned to a great extent by many of our members' being in active military service and the fact that others are deluged with research connected with the war effort, while the clinicians are overwhelmed with private practice.

HENRY H. TURNER, M.D., *Secretary-Treasurer*

ERRATA

THE FOLLOWING corrections and additions pertain to the paper entitled "The Effect of Thyroid Treatment on the Respiration of Various Rat Tissues" by Edgar S. Gordon and Arthur E. Heming which appeared in ENDOCRINOLOGY, volume 34, number 6, pages 353–360. (Editors Note.)

[1] This data was presented as a partial fulfillment of the requirements of the University of Wisconsin for the degree of Doctor of Philosophy, June, 1941 (AEH).

[2] Present address—Johnson and Johnson do Brasil, Caixa Postal, 136-A, São Paulo, Brasil.

TABLE 1. COMPARISON OF THE EFFECT OF THYROXINE INJECTIONS[1] ON THE RESPIRATION OF VARIOUS RAT TISSUE SLICES

Tissue	Normal		Time After Initial Injection (in days)											
			2			3			4			6		
	Q_{O_2}	No. of animals	Q_{O_2}	% Inc.	No. of animals	Q_{O_2}	% Inc.	No. of animals	Q_{O_2}	% Inc.	No. of animals	Q_{O_2}	% Inc.	No. of animals
Liver	5.6	25	7.7	37.5	6	7.9	41.0	10	9.1	62.4	13	7.0	25.0	6
Kidney[2]	22.7	23	29.5	29.9	7	29.7	30.8	10	31.3	37.8	9	27.0	18.9	8
Testis	5.5	4	5.0	9.1	6	5.6	3.6	9	5.3	3.6	7			
Heart	3.3	18	3.4	3.0	5	4.1	24.2	9	2.9	12.1	9	2.6	21.1	8
Brain[2]	11.2	21	11.7	4.3	7	10.9	2.8	10	10.7	4.3	9	10.8	3.6	8
Spleen	11.1	14	12.3	10.8	1				11.2	0.9	2	10.6	4.5	8
Diaphragm	5.6	10	6.4	14.3	6	7.2	28.6	6	7.2	28.6	5	6.7	19.6	4

Values represent averages of duplicates from the number of animals indicated.

[1] 20 mg. of dl-thyroxine per kilo of body weight was injected on two successive days.

[2] In presence of 0.2% glucose.

TABLE 2. EFFECT OF THYROID FEEDING[1] ON THE RESPIRATORY QUOTIENT OF RAT LIVER AND DIAPHRAGM

Tissue	Substrate	Normal		Hyperthyroid	
		R.Q.	No. of animals	R.Q.	No. of animals
Liver Slice	None	0.83	9	0.73	9
Kidney Slice	0.2% Glucose	0.89	8	0.94	9
Diaphragm Strips	None	0.89	9	0.77	15
Liver Slice	0.2% Glucose	0.82	2	0.72	2
Liver Mince	None	0.50	5	0.46	4
Liver Mince	0.2% Glycogen	0.49	5	0.45	4

Values represent averages of duplicates from the number of animals indicated.

[1] 1 gm. of desiccated thyroid per kilo of body weight per day was fed for three successive days.

TABLE 4. RESPIRATION OF NORMAL AND HYPERTHYROID[1] RAT TISSUE SLICES IN THE PRESENCE OF VARIOUS SUBSTRATES[2]

Tissue	0.02 M Substrate	Q_{O_2}		% Difference	
		N	H	N	H
Liver	None	6.6	8.1	—	—
Liver	Succinate	10.5	12.4	59.0	53.1
Liver	Citrate	7.3	8.7	10.6	7.4
Liver	None	7.0	8.1	—	—
Liver	Malate	8.7	9.5	24.3	17.3
Liver	Fumarate	7.6	9.0	8.6	11.1
Kidney	None	27.2	33.5	—	—
Kidney	Succinate	46.6	45.0	71.1	34.4
Kidney	Fumarate	31.4	37.6	15.4	12.6
Kidney	None	27.4	33.4	—	—
Kidney	Malate	30.5	35.9	11.3	7.5
Kidney	Citrate	33.4	38.4	22.0	15.0
Diaphragm	None	6.1	7.6	—	—
Diaphragm	Succinate	8.2	8.1	34.4	6.6
Diaphragm	None	5.9	7.2	—	—
Diaphragm	Fumarate	6.2	7.8	5.1	8.4

[1] Hyperthyroidism was produced by feeding 1 gm. of desiccated thyroid U.S.P per kilo of body weight per day for three days.

[2] Results from 2–6 animals.